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AMINO ACID COMPOSITION OF ALKALI-INSOLUBLE PROTEINS OF DIPHTHERIA MICROBES

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Investigation of the amino acid composition of bacterial cells has led to the discovery of a number of "new" amino acids which are not usually encountered in animal and plant material. This discovery has been assisted by new methods of investigation of amino acid composition and, more particularly, by the method of partition chromatography on paper.

Aminoadipic and hydroxyaminoadipic acids have been found in Vibrio cholerae [1]. Aminoadipic acids with six carbon atoms constitute about 2.5% of the dry weight of bacteria. An amino acid has been found in the toxin which was isolated from a filtrate of Pseudomonas tabaci culture [2]; this amino acid proved to be $\alpha_1\epsilon$-diamino-β-hydroxypimelic acid, which constituted the greater part of the toxin molecule. Approximate data concerning the presence of α-amino-n-butyric acid and α-amino-n-heptylic acid in the hydrolyzate of E. coli have been reported by Polson [3].

A "new" amino acid has been found in diphteria microbes [4]. It has been isolated in the crystalline state and identified as $\alpha_1\epsilon$-diaminopimelic acid. Further investigations showed that this amino acid was widely distributed among microbes.

We did not succeed in establishing the presence of diaminopimelic acid in the course of our studies [5] of the amino acid composition of alkali-soluble proteins from diphtheria microbes. The proteins concerned were extracted from defatted bacterial cells by 4% solution of caustic soda. However, considerable amounts of bacterial residues remained after extraction of alkali-soluble proteins.

The present work is concerned with studies of the amino acid composition of this bacterial residue.

EXPERIMENTAL

The starting material was a culture of Corynebact. diphtheriae strain PW-8. The microbial mass was preliminarily extracted with organic solvents – ether, acetone, chloroform. The defatted cells were extracted with 4% NaOH. The bacterial residue left after alkaline extraction constituted the greater part of the bacterial cells by weight – approximately 80% of the dry weight of defatted bacterial mass.

The amino acid composition of the bacterial residue was studied first. 200 mg of the substance was hydrolyzed with 6 N HCl, after which the hydrolyzate was diluted with distilled water and filtered to remove humic substances; the filtrate was then evaporated to dryness under vacuum. This evaporation and solution procedure was repeated thrice in order to remove as much hydrochloric acid from the hydrolyzate as possible. The hydrolyzate thus prepared was subjected to chromatographic separation. Since large fluctuations in the quantitative content of individual amino acids are possible in a given hydrolyzate, some doubt could arise concerning the complete demonstration of all the amino acids. Repeat experiments were therefore carried out with separation of the amino acids on paper, upon application of different amounts of the hydrolyzate.

Chromatographic separation of the amino acids was achieved by two-dimensional ascending paper chromatography. A tall vacuum desiccator served as a chromatographic analysis chamber; its lid was replaced by a glass bell with a ground surface. A Koch dish was placed on the bottom of this desiccator and filled with mobile solvent.

The free space between the Koch dish and the walls was filled with a mixture which served to saturate the atmosphere within the desiccator. The sheet of filter paper was rolled into a tube and maintained in this way by means of a special glass holder. Phenol saturated with water and butyl-acetic* mixture saturated with water were used as solvents. Butyl-acetic mixture was used as the first solvent. When phenol was used the atmosphere within the chamber was additionally saturated with ammonia vapor (0.03%). To ensure better partition twice-repeated passage of butyl-acetic mixture in the same direction was used in isolated cases. Detection of the amino-acids on paper was achieved by using 0.1% solution of ninhydrin in water-saturated butyl alcohol.

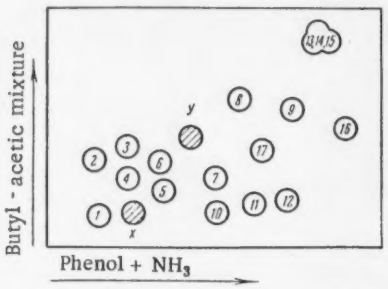

Fig. 1. Diagram of the chromatogram given by hydrolyzate of bacterial residue:
1) Cystine; 2) aspartic acid; 3) serine; 4) glutamic acid; 5) glycine; 6) threonine; 7) alanine; 8) tyrosine; 9) valine; 10) lysine; 11) arginine; 12) histidine; 13), 14), 15) leucine, isoleucine, phenylalanine; 16) proline; 17) aminobutyric acid; X, Y) unidentified substances.

The two-dimensional chromatogram obtained during investigation of the hydrolyzate of alkali-insoluble protein of diphtheria microbes revealed 19 spots which corresponded to the following 17 amino-acids: aspartic acid, cystine, serine, glutamic acid, glycine, alanine, threonine, valine, lysine, arginine, histidine, α-amino-butyric acid, leucine, isoleucine, phenylalanine, tyrosine and proline (Fig. 1). Two spots X and Y were not identified.

It was supposed that diaminopimelic acid might be present in the material under investigation, and not having a standard sample of this amino acid, we decided to isolate it. The eluate of spot X was applied to a strip of filter paper which was then placed in the electrophoresis chamber [6]. The substance of this eluate proved to be electrically neutral, i.e., it remained at the site of application without moving either to the cathode or the anode.

Subsequently, the neutralized hydrolyzate of the bacterial residue, containing a mixture of various amino acids, was separated by means of electrodialysis.

Separation was effected in an electrodialyzer. The latter consisted of a three-chamber ebonite apparatus. The dimensions of its sections were 10 × 10 × 1.5 cm. It was possible to achieve separation in this apparatus using 25 ml of the hydrolyzate. Platinum wire served as electrodes. In this case the anode and cathode solutions were colorless. Direct current of 100 v voltage was applied to the electrodes which were in the lateral sections of the dialyzer. The current strength increased during the experiment, reached a maximum of 600 ma and then fell to 50-100 ma. Under our experimental conditions electrodialysis was usually finished after 1 hour. Chromo-gelatin membranes [7] were used between the compartments of the apparatus. Such membranes are the most suitable and give sufficiently clear separation of the amino acids into the major groups: monoamino-acids, dicarboxylic acids and diamino-acids. Separation of the amino acids into these groups was monitored by chromatography. The results obtained were highly satisfactory.

For further experiments the solution from the middle section of the electrodialyzer was used; it consisted of a mixture of neutral amino acids.

The mixture of neutral amino acids was subjected to partition in a column of alumina (height of column 50 cm, diameter 7.0 mm). The alumina was first treated with hydrochloric acid (4 g of the adsorbent agent was treated with 12 ml N HCl, shaken for 5 minutes and allowed to stand for 10-15 minutes). The alumina was then washed with distilled water until a neutral reaction was obtained to litmus. 4 g of the adsorbent material was used for partition of a mixture containing 6 mg NH_2-group nitrogen. Solution of neutral amino acids was sucked through the column with the help of a manual vacuum pump at a rate of 30 drops per minute. The column was washed with 50 ml water saturated with hydrogen sulfide in order to remove cystine and then with another 50 ml water (distilled). After this, 3 ml 3N NH_4OH was added to the column and the column washed with 30 ml 0.5 N NH_4OH. The alkaline eluate was collected and evaporated to dryness under vacuum. The dry residue was dissolved in absolute ethyl alcohol. Solution was not, however, complete. The undissolved part was filtered off and absolute alcohol was added repeatedly to the filtrate. White crystals precipitated out on gradual evaporation of the alcoholic solution. They were recrystallized three times.

* Butanol-acetic acid.

Kjeldahl determination of nitrogen on the crystalline product obtained gave a value of 14.66%; theoretical nitrogen content of diaminopimelic acid 14.73%, sample 10.5 mg (11.0 ml 0.01 N acid taken up).

The substance obtained was also examined by paper chromatography. Rhuland, Work, Denman and Hoare [8] have shown that when the solvent used consists of the following mixture: methanol – water – 10 N hydrochloric acid – pyridine (80 : 17.5 : 2.5 : 10) all the amino acids with the exception of diaminopimelic acid isomers move very rapidly behind the front of the solvent; diaminopimelic acid lags very markedly in its movement. When the substance obtained by us was subjected to this treatment it was found that it moved very slowly along the paper, lagging behind the other 16 standard amino acids used in the experiment. When the preparation obtained was added to the hydrolyzate of the bacterial residue there was enhancement of the color of spot X on the two-dimensional chromatogram of the hydrolyzate.

The data on nitrogen content and behavior on paper chromatographing with the solvent methanol – water – – 10 N hydrochloric acid – pyridine thus provide evidence that the crystalline preparation obtained from the bacterial cell residue is diaminopimelic acid.

SUMMARY

A study was made of the amino acid composition of bacterial cell residue of Corynebacterium diphtheriae after preliminary defatting and extraction with 4% solution of alkali.

Partition chromatography on paper revealed the presence of 17 amino acids in the hydrolyzate of the bacterial residue, including: aspartic acid, cystine, serine, glutamic acid, glycine, threonine, alanine, tyrosine, valine, lysine, arginine, histidine, leucine, isoleucine, phenylalanine, proline and α-aminobutyric acid. One of the two unidentified spots on the chromatogram corresponded to diaminopimelic acid. By means of electrodialysis and subsequent partition in an alumina column a crystalline substance was obtained which was identified as diaminopimelic acid on the basis of quantitative nitrogen estimation and chromatographic behavior in a solvent consisting of methanol – water – hydrochloric acid – pyridine.

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**In Russian.

THE EFFECT OF SALICYLIC AND PARA-AMINOSALICYLIC ACIDS ON THE SYNTHESIS OF PARA-AMINOHIPPURIC ACID BY RAT LIVER SLICES

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The liver and kidneys contain an enzyme system which synthetizes hippuric acid from benzoic acid and glycine, and also p-aminohippuric acid (PAHA) from p-aminobenzoic acid (PABA) and glycine.

The synthesis of the former substance has been described by Borsook and Dubnoff [1], that of the latter by Cohen and Gilvery [2].

According to Cohen and Gilvery [2], the synthesis of both compounds is effected by the same enzyme system since benzoic acid inhibits competitively the synthesis of PAHA. The enzyme activity of this system is associated with large intracellular particles. Kielley and Schneider [3] have shown that the enzymes taking part in the synthesis of PAHA are associated with mitochondria. Cohen and Gilvery [2] have established that the energy required for the synthesis under aerobic conditions is supplied by oxidative reactions of the tricarboxylic acid cycle and under anaerobic conditions by adenosine triphosphate (ATP) breakdown. According to these authors, the process of PAHA synthesis is inhibited by cyanides, arsenites, iodoacetate, azide, malonate, sodium fluoride. Formation of PAHA by rat liver homogenates is optimal in the presence of magnesium ions, potassium ions, cytochrome and citric acid cycle metabolites.

The enzyme system under consideration is highly specific: glycine cannot be replaced by another amino acid or peptide.

Braunshtein and Efimochkina [4] showed that PAHA synthesis in rats declines progressively on a diet deficient in pantothenic acid and is restored after one or two injections of the latter. The authors put forward the suggestion that coenzyme A (CoA), of which pantothenic acid is a constituent, takes part not only in acetylation, but also in the synthesis of amide and peptide linkages.

Chantrenhe [5] studied the enzymatic benzoylation of glycine and found that under anaerobic conditions synthesis of hippuric acid occurred only in the presence of CoA and ATP. The author removed CoA from the enzyme extract by means of ion-exchange adsorbent agent and restored the process of hippuric acid formation by addition of CoA in high concentrations (40 μg/ml).

Ivanovics [6] studied the mechanism of the bacteriostatic action of salicylic acid with respect to Staphylococcus aureus. The author succeeded in showing that the sodium salt of salicylic acid in 0.001 M concentration completely suppressed the growth of Staphylococcus aureus in synthetic medium and that the action of 0.005 M salicylate was completely abolished on addition of 10^{-7} M pantothenic acid. None of the other biologically important compounds tested showed this property. Ivanovics considers the salicylate ion to be a specific inhibitor of the biosynthesis of pantothenic acid.

On the basis of the reviewed literature data we considered it important to study the effect of salicylic acid on PAHA synthesis by rat liver slices from PABA and glycine. We also studied the effect on this reaction of one of the salicylic acid derivatives, viz., p-aminosalicylic acid, which is an antituberculosis chemotherapeutic agnet.

The procedure described by Cohen and Gilvery [2] was used in the present work. The studies were carried out in vitro with simultaneous incubation of substrate and of preparations with liver slices over a period of 5 hours.

EXPERIMENTAL RESULTS

The experimental results, presented in Table 1, show that salicylic acid inhibits significantly the synthesis of p-aminohippuric acid. In concentration of 1 : 1000 this inhibition is complete (100%), in concentration 1 : 2000 it reaches 97%. Inhibition also takes place when concentrations of 1 : 10,000 and 1 : 100,000 are used (by 80 and 28%). Inhibition is only absent when the concentration used reaches 1 : 1,000,000.

TABLE 1

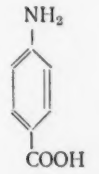
Effect of Salicylic Acid on PAHA Synthesis by Rat Liver Slices

Amount of tissue in all experiments 0.2 g

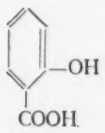
Salicylic acid concentration in %	Synthesis in the presence of salicylic acid in μg/5 ml	Synthesis without salicylic acid in μg/5 ml	Inhibition of PAHA synthesis in %	Average inhibition in %
0.1	0	722	100	
0.05	8.5	543	98.5	
0.05	8.5	552	98.5	97
0.05	40.0	793	95	
0.01	128.0	722	83	
0.01	125.0	543	78	
0.01	122.0	552	78	80
0.01	160.0	793	79	
0.01	155	766	80	
0.002	420	766	46	
0.001	436	543	20	
0.001	515	793	36	28
0.001	436	552	21	
0.001	493	766	37	
0.0001	557	543	0	
0.0001	557	552	0	

Data presented in Table 2 show the inhibitory action of p-aminosalicylic acid on the synthesis of p-aminohippuric acid. Interference with the synthesis is less marked in this case than in the experiments with salicylic acid.

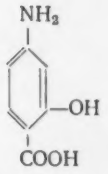
The data obtained by us are, in our opinion, of theoretical interest. Suppression of synthesis in this case may depend on the fact that salicylic and p-aminosalicylic acids are close in their chemical structure to one of the substrates of the enzymatic reaction, viz., p-aminobenzoic acid.



Para-aminobenzoic acid (substrate)



Salicylic acid (inhibitory substance)



Para-aminosalicylic acid (inhibitory substance)

Similar interrelations may exist in this case as in the inhibition of succindehydrogenase by malonic acid. A similar mechanism of action is observed also in microbial cells in the course of sulfonamide or PASA inhibition of metabolic processes in which PABA participates when sulfonamide or PASA is incorporated instead of PABA into formylfolic acid, and disturbances of biochemical reaction in the organism ensue as the result.

SUMMARY

Salicylic acid in concentrations of 0.001-0.1% inhibits the reaction of p-aminohippuric acid formation from

p-aminobenzoic acid and glycine by liver slices; the inhibition amounts to 30-100%. Para-aminosalicylic acid also inhibits this biosynthesis but to a markedly smaller degree than salicylic acid.

TABLE 2

Effect of Sodium Salt of P-Aminosalicylic Acid on PAHA Synthesis by Rat Liver Slices

PASA concentration in %	Amount of tissue in g	Synthesis in the presence of PASA (in μg/5 ml)	Synthesis without PASA (in μg/5 ml)	Inhibition of synthesis in %	Average inhibition in %
0.1	0.5	377	552	34	
0.1	0.5	568	934	32	
0.1	0.2	268	383	32	41
0.1	0.1	86	138	38	
0.1	0.2	368	712	48	
0.1	0.1	115	305	62	
0.1	0.2	383	675	44	
0.05	0.2	477	675	30	
0.01	0.2	455	712	36	32
0.01	0.1	215	305	30	
0.001	0.2	357	383	6	
0.001	0.1	86	138	38	20
0.001	0.1	242	305	21	
0.001	0.2	609	712	15	
0.0001	0.2	651	712	9	
0.0001	0.1	242	305	21	15

Inhibition of the biosynthesis of p-aminohippuric acid by salicylic and p-aminosalicylic acids may be explained by competition of these inhibitory substances with the substrate.

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THE EFFECT OF BARBITURATES ON THE ACTIVITY OF CEREBRAL ASPARAGINASE AND GLUTAMINASE

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The aim of the present work was the study of changes in the activity of asparaginase and glutaminase in the brain of white rats under the influence of barbiturates, which elicit inhibition of the central nervous system.

Asparaginase and glutaminase are specific enzymes which deaminate asparagine and glutamine with formation of ammonia, and aspartic and glutamic acids, respectively. These acids and their amides – asparagine and glutamine – play an important role in nitrogen metabolism and participate actively in the processes which render ammonia harmless in the body.

A number of authors [1-12] have investigated the activity of the enzymes glutaminase and asparaginase under various physiologic and pathologic conditions. However, in the literature available, we have not encountered any data on the effect of neurotropic substances on the above-mentioned enzyme systems.

EXPERIMENTAL METHOD

White rats served as experimental animals. The asparaginase and glutaminase activity was determined by the Mardashev and Lestrovaia method [1]. The activity of these enzymes was judged by the amount of ammonia formed as the result of deamination of asparagine and glutamine under the influence of the appropriate enzymes.

A chemically pure preparation of asparagine was used; glutamine was obtained from the roots of fodder beets by the method of Vickery, Pucher and Clark [13]. The purity of the preparations was checked by total nitrogen determination (micro-Kjeldahl method) and by paper-chromatographic analysis[14].

For the determination of ammonia, into one half of the outer chamber of the Conway vessel was introduced 1 ml phosphate buffer (pH 8.0), 1 ml 0.01 M aqueous solution of asparagine or glutamine, 1 ml of brain homogenate. Into the other half was placed 2 ml saturated aqueous solution of K_2CO_3. Into the inner chamber was measured 1.2 ml 0.01 N H_2SO_4 with three drops of Tashiro indicator [15]. After this the vessel was quickly covered with a heated glass and it was incubated at room temperature for 3 hours. After 3 hours the contents of the outer compartments were mixed by rhythmic rotary movements, thus terminating the enzymatic reaction. 24 hours after mixing of the solutions in the outer compartment the remaining 0.01 N H_2SO_4 was titrated with 0.01 N NaOH.

In order to investigate changes in the enzymes during brief and prolonged sleep and under anesthesia, medinal and sodium amytal were used as hypnotics; these were injected as freshly prepared solutions subcutaneously into the rats' thighs. For brief sleep medinal was administered on the basis of 15 mg per 100 g body weight in the form of 4% aqueous solution once a day. For prolonged sleep medinal was given in the same dose over a period of 5 days (twice daily). In order to induce sleep close to anesthesia, the rats were given medinal as 10% solution, 30 mg per 100 g body weight. Sodium amytal was given as 1% solution, 3.3 mg per 100 g body weight, once a day (during one day) for brief sleep, and in the same dose during 5 days twice a day for prolonged sleep. Sleep approaching narcosis was induced in rats by administration of sodium amytal as 2% solution on the basis of 8 mg per 100 g body weight.

Medinal induced sleep in rats, lasting 5 ½ -6 hours, sodium amytal – 2 ½ -3 hours. In short-term experiments with medinal sleep we investigated the activity of asparaginase and glutaminase after one-, two-, four- and six-

hour sleep, in experiments with sodium amytal after one-, two- and three-hour sleep.

RESULTS

The experimental data obtained, shown in the table, reveal that the activity of asparaginase in the brain of healthy rats varies within the range of 10 to 15 μg per 1 ml homogenate (average 11 μg), and the activity of glutaminase is higher and varies from 12 to 31 μg (average 21.16 μg) per 1 ml homogenate. Our data on the activity of asparaginase in the brain of healthy rats agree with the data obtained by Mardashev and Lestrovaia [1].

Asparaginase and Glutaminase Activity in White Rat Brain in the Normal State, During Brief Sleep, Prolonged Sleep and Narcosis

Experimental conditions	Asparaginase		Glutaminase	
	No. of analyses	Activity	No. of analyses	Activity
Healthy rats	33	11.0 (10.0—15.0)	30	21.16 (12.0—31.0)
One-hour medinal sleep	10	4.9 (2.0—7.0)	8	16.0 (9.0—27.0)
Two-hour medinal sleep	10	2.3 (2.0—3.0)	8	16.0 (6.0—27.0)
Four-hour medinal sleep	10	1.1 (0.0—2.0)	11	16.8 (10.0—23.0)
Six-hour medinal sleep	8	4.6 4.0—7.0)	8	13.0 (11.0—17.0)
One-hour sodium amytal sleep	8	1.5 (0.0—5.0)	8	18.0 (8.0—30.0)
Two-hour sodium amytal sleep	8	(3.3 0.0—8.0)	8	18.0 (10.0—28.0)
Three-hour sodium amytal sleep	8	4.1 (0.0—7.0)	8	21.0 (10.0—30.0)
Prolonged medinal sleep	10	2.1 (0.0—5.0)	10	16.0 (9.0—23.0)
Prolonged sodium amytal sleep	9	0.8 (0.0—2.0)	9	16.0 (13.0—22.0)
Medinal narcosis	8	3.6 (0.0—9.0)	8	20.5 (16.0 – 25.0)
Sodium amytal narcosis	8	2.2 (0.0—8.0)	8	18.5 (14.0—22.0)

Note 1. Enzyme activity is expressed in μg N-NH_3 per 1 ml homogenate (homogenate in 1 : 3 dilution). 2. Mean values and extreme figures (in parentheses) are given.

The experiments mentioned showed that during brief medinal-induced sleep there was considerable inhibition of asparaginase activity, its greatest inhibition occurring after four-hour sleep, which corresponded to the greatest depth of sleep. In the same experiments the activity of glutaminase was lowered to a lesser degree than that of asparaginase.

During brief sodium amytal-induced sleep an almost analogous picture was observed, viz.,the asparaginase activity was decreased very sharply, while the glutaminase activity diminished only slightly. Unlike medinal-induced sleep, sodium amytal sleep produced the greatest decrease of asparaginase activity after one-hour sleep which coincided in time with the greatest cerebral inhibition elicited by sodium amytal action.

During prolonged (5 days) medication-induced sleep there was even more marked decrease in asparaginase

activity than in the short-term experiments and some lowering of glutaminase activity.

The results of the experiments with the use of narcotic doses of sodium amytal and medinal show that asparaginase activity drops very sharply, whereas there is no substantial decrease in the activity of glutaminase in this case either. These data indicate that glutaminase activity, even under conditions of narcosis, remains at a higher level than the activity of asparaginase.

DISCUSSION

The results of our investigations have shown that the activity of the enzymes studied changes depending on the duration and strength of action of the barbiturates. This was particularly clearly shown for asparaginase, whose activity decreased sharply even during the first few hours of sleep and was more sharply inhibited the deeper and more prolonged the sleep. On the appearance of the first signs of waking the value of asparaginase activity again increased, but did not reach the initial values at the time of awakening. No such consistent features could be noted with respect to glutaminase.

The results of the experiments described thus show that during inhibition of the central nervous system an acute decrease of asparaginase activity and some decrease of glutaminase activity take place. Inhibition of the central nervous system produced by medication-induced sleep is characterized by a decrease in the content of ammonia which forms as the result of asparagine and glutamine deamination.

Our data again confirm the hypotheses postulated by Palladin [16], Vladimirov [17] and Vladimirova [18] that inhibition is an active process, characterized by rapid removal of excess ammonia in the brain.

SUMMARY

A study was made of the activity of asparaginase and glutaminase in the brain of white rats in the normal state, during brief and prolonged sleep and during narcosis. In all cases of medication-induced sleep, effected by subcutaneous injections of medinal and sodium amytal, the activity of the enzymes under investigation underwent marked changes. Decrease of asparaginase activity was particularly marked, the decrease being the greater the deeper and more prolonged the sleep. Asparaginase activity showed an increase upon the first signs of the animal's waking.

Throughout the period of sleep the activity of glutaminase decreased, but less markedly than that of asparaginase.

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THE INTERRELATION OF RIBONUCLEIC ACID AND THE ACTIVITY OF RIBONUCLEASE AND PROTEASE WITH TOXIN FORMATION IN C. DIPHTHERIAE

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The known relation between changes in nucleic acids and the activity of enzymes is important biologically in the study of metabolism and biosynthesis of proteins or protein-like compounds, including some bacterial toxins.

Dernby and Walbum [1] suggested a relationship between toxin formation of C. diphtheriae and protease activity; we established this interrelationship [2] under different conditions of cultivation of C. diphtheriae.

Several authors [3, 4] showed that pancreatic ribonucleic acid (RNA) blocks the proteolytic activity of trypsin and cathepsin. We [5] also found a blocking action by C. diphtheriae RNA on the activity of protease in vitro; while under the influence of yeast RNA the hydrolytic function of C. diphtheriae proteolytic enzymes was strengthened.

In this connection we decided to study the interrelation between RNA, ribonuclease, and protease activity of C. diphtheriae in the biosynthesis of diphtheria toxin. We found nothing in the literature which related to this question.

EXPERIMENTAL METHODS

C. diphtheriae, Strain PW-8, was cultured on meat-peptone broth (pH 7.8) for five days in a microfermentation apparatus [2]. Three sets of experiments were run simultaneously: 1 – control, 2 – experiments with activation of ribonuclease by calcium, and 3 – with blocking of ribonuclease by sodium azide [6].

Calcium (10^{-2} M) and sodium azide (10^{-2} M) were added to the medium on the second day of culturing C. diphtheriae. Calcium was used in the form of $CaCO_3$, since this substance is used in preparing diphtheria toxin to maintain the optimum pH for toxin formation.

Ribonuclease activity was determined by the method of Muggleton and Webb [7] in the microbe mass after washing three times with physiological saline, and also in the culture medium after filtration through a Zeiss filter and lyophilic drying. The RNA content was determined by the method of Schneider [8]. Protease activity in the microbe mass was determined by comparison of the increase in amino nitrogen after autolysis with the total nitrogen, using the micro-Kjeldahl method. Inorganic (P_i) and organic (P_o) phosphorus were determined by the method of Fiske-Subbarow. Enzyme activity, RNA content, inorganic and organic phosphorus were expressed in percent of the absolutely dry substance.

The contents of RNA, inorganic, and organic phosphorus in the culture medium (Figs. 2, 4, and 6) were expressed in percent of these substances in the starting medium. The titer of diphtheria toxin was determined by the method of Ramon by flocculation with diphtheria serum antitoxin and expressed in L_f.

EXPERIMENTAL RESULTS

Fig. 1 shows that in the control experiments on toxin formation by C. diphtheriae, there was an increase in ribonuclease activity and a decrease in RNA content in the microbe mass.

Since McCarty [9] found RNA-ase in the culture medium during bacterial growth, we also studied the activity

of this enzyme in the culture medium.

It was interesting to observe that analogous changes occurred in the culture medium, namely: rise in ribonuclease activity of C. diphtheriae and a gradual fall in RNA content (Fig. 2).

The data of Fig. 1 show that the fall in RNA content in the bacterial mass was accompanied by a lessened hydrolytic function of protease; this effect evidently was produced by the action of ribonucleic acid, since RNA, as several authors [3, 4] and we [5] showed, blocked the hydrolytic functionof C. diphtheriae protease in experiments in vitro. Such a decrease in proteolytic activity of C. diphtheriae was very possibly accompanied in our experiments by an increase in diphtheria toxin titer to 45 L_f.

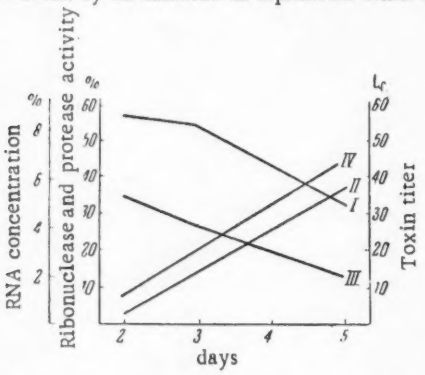

Fig. 1. Change in RNA content, activity of RNA-ase and protease in C. diphtheriae microbe mass during toxin formation (control experiment).
I) RNA; II) ribonuclease activity; III) protease activity; IV) toxin titer.

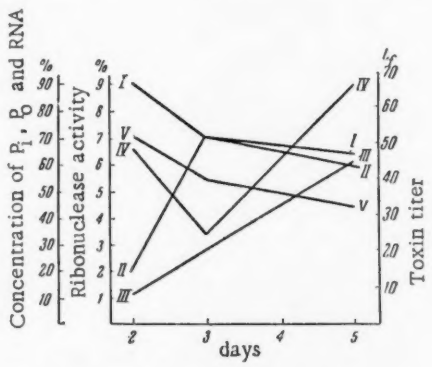

Fig. 2. Change in organic and inorganic phosphorus content, RNA, and RNA-ase activity in the culture medium during toxin formation by C. diphtheriae (control experiment).
I) RNA; II) RNA-ase activity; III) toxin titer; IV) organic phosphorus; V) inorganic phosphorus.

We studied changes in the content of inorganic and organic phosphorus in order to investigate variations in total phosphorus compounds of the medium during toxin formation by C. diphtheriae. As Fig. 2 shows, when there was a gradual decrease in inorganic phosphorus content in the culture medium, there was also some decrease in organic phosphorus up to the third day of culturing the C. diphtheriae, and then it increased.

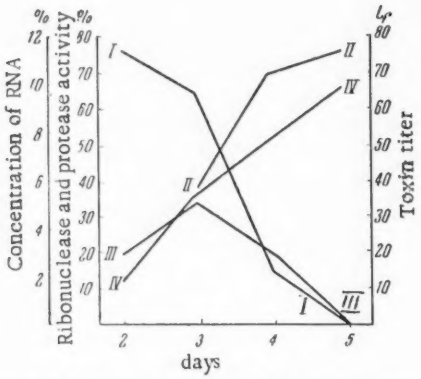

Fig. 3. Change in content of RNA, activity of RNA-ase and protease in the microbe mass of C. diphtheriae during toxin formation (Ca added to the culture medium).
I) RNA; II) RNA-ase activity; III) protease activity; IV) toxin titer.

Thus, a decreased content of RNA in these experiments was accompanied by a fall in the amount of inorganic phosphorus and a rise in organic phosphorus; this rise was evidently related not only to increased nucleotide content from hydrolysis of RNA by ribonucleotidase, but also to the synthesis of organophosphorus compounds. We observed a rise in the diphtheria toxin titer along with these changes in the content of phosphorus compounds in the culture medium.

The data from these experiments suggested the participation of these components of the microbe cell in toxin formation by C. diphtheriae (Figs. 1 and 2).

To confirm this, we ran experiments with activators and inhibitors of C. diphtheriae ribonuclease.

The data of Fig. 3 show that during toxin formation in the bacterial mass, ribonuclease activity increases, accompanied by a sharp fall in the amount of RNA and proteolytic activity of C. diphtheriae.

A series of experiments in vitro showed that addition of calcium increased protease activity by 20-30%. Evidently, in the experiments in vivo also, the rise in pro-

tease activity occurred under the influence of calcium which was added on the second day of culturing the C. diphtheriae. The sharp fall in proteolytic activity of C. diphtheriae at the end of toxin formation was probably connected with the blocking action of RNA.

When we compared these experiments with the controls we observed a greater change in ribonuclease and protease activity and in RNA content in the microbe mass, accompanied by an increase in diphtheria toxin titer to 65 L_f.

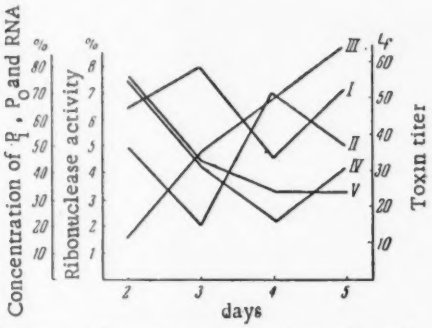

Fig. 4. Change in content of organic and inorganic phosphorus, RNA, and RNA-ase activity in the culture medium of C. diphtheriae during toxin formation (Ca added to the culture medium).
I) RNA; II) RNA-ase activity; III) toxin titer; IV) P_o; V) P_i.

Thus the data of these in vivo experiments indicated an influence of ribonuclease and RNA on change in the hydrolytic function of C. diphtheriae protease during biosynthesis of diphtheria toxin.

In the culture medium (Fig. 4) the change in ribonuclease activity was accompanied by a corresponding variation in RNA content; it should be remarked that at the end of toxin formation by C. diphtheriae there was some increase in RNA content with decreased ribonuclease activity; this evidently depended on secondary changes which occurred both in the medium and in the microbe cell. This latter effect probably occurs because of increased autolysis by the microbial cell at this time and liberation of RNA from C. diphtheriae in connection with the weakened ribonuclease activity [10].

When we compared the changes in inorganic and organic phosphorus in the culture medium with the control experiments, we found a greater decrease in amount of inorganic phosphorus and an increase in organic phosphorus content which occurred from the fourth day of culturing C. diphtheriae, along with a rise in RNA content.

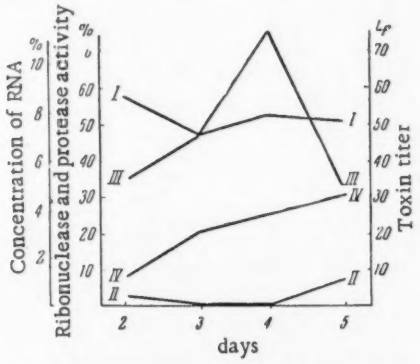

Fig. 5. Change in RNA content, activity of RNA-ase and protease of the C. diphtheriae microbe mass during toxin formation (sodium azide added to the culture medium).
I) RNA; II) activity of RNA-ase; III) activity of protease; IV) toxin titer.

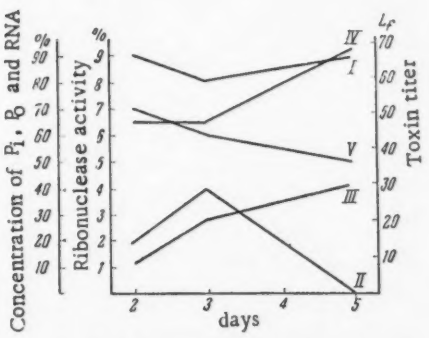

Fig. 6. Change in content of organic and inorganic phosphorus, RNA, and activity of RNA-ase in the culture medium during toxin formation by C. diphtheriae (sodium azide added to the culture medium).
I) RNA; II) RNA-ase activity; III) toxin titer; IV) P_o; V) P_i.

The results of these investigations again showed the share of ribonuclease, protease, and RNA in toxin formation and were also confirmed by the experiments with inhibiting the ribonuclease with sodium azide.

As the data in Fig. 5 show, inactivation of the ribonuclease in the microbe mass stops the culturing of C. diphtheriae. In this connection it was also necessary to remark that changes in RNA content when ribonulease was inhibited by sodium azide were small; the proteolytic activity of C. diphtheriae was at a very high level for four

days of culturing, after which it began to fall. We should mention that sodium azide in the control experiments in vitro had no effect on the protease activity of C. diphtheriae.

The results of the experiments on inhibiting the ribonuclease and maintaining the RNA almost unchanged indicate that RNA in the microbe cell evidently occurs with the same degree of polymerization and therefore can inhibit proteases equally. Various complexes are thus formed which differ from each other in their structures and physicochemical properties, and this in turn is reflected in changes in enzyme activity of the proteolytic enzymes and in the biosynthesis of diphtheria toxin. The high proteolytic activity of C. diphtheriae which we found in the experiments with inhibition of ribonuclease is accompanied by slight increase in titer of the diphtheria toxin to 30 L_f, and it evidently causes partial decomposition of the toxin; according to the literature [11] proteolytic enzymes decompose diphtheria toxin.

In the culture medium, the RNA content (Fig. 6) was almost unchanged, except on the third day of toxin formation by C. diphtheriae; then there was some fall in RNA along with an increase in ribonuclease activity. When the ribonuclease was inhibited by sodium azide, the change in content of inorganic and organic phosphorus changed in the same way that these substances changed in the control experiments.

Thus, the results of this investigation show some participation of RNA and ribonuclease in the changes of protease activity in the biosynthesis of diphtheria toxin.

SUMMARY

When we study the change in content of ribonucleic acid (RNA), and the activity of ribonuclease and protease during toxin formation by C. diphtheriae, we find participation by these components in the biosynthesis of this toxin; this has been confirmed by experiments with activation and inhibition of ribonuclease.

The increased activity of ribonuclease in the presence of calcium is accompanied by a sharp fall in RNA content, and proteolytic activity of C. diphtheriae, and increase in titer of diphtheria toxin in the culture medium.

When the activity of ribonuclease is inhibited by sodium azide and the content of RNA remains almost at the original level, there is a sharp rise in hydrolytic function of the protease of C. diphtheriae and a decreased biosynthesis of diphtheria toxin.

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THE PROTEINASE OF CL. OEDEMATIENS

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Investigators [1, 2] who have studied the proteolytic power of microorganisms which cause gas gangrene (a group which includes Cl. oedemations) have come to the conclusion that these microbes possess a proteinase with an optimum pH 7 which is activated by SH compounds. So far there has been no direct study of Cl. oedematiens. Fermi [3] concluded from indirect evidence that a proteinase of Cl. oedematiens existed because of the decomposition of the protein substrate on which the culture was grown. He showed that Cl. oedematiens easily decomposed casein and gelatin, and less easily, fibrin. The microbe did not grow at all on egg albumen and blood serum in the experiments of Fermi. Smith and Lindsley [4] showed that when Cl. oedematiens was added to a medium of normal rabbit serum it did not lose its hydrolyzing power toward gelatin, in distinction to a number of other organisms of the clostridia type.

The absence of literature data showing directly the presence of proteolytic enzymes in Cl. oedematiens is explained partly by the fact that all studies on bacterial proteases have been conducted with enzymes which the microbes give off to the medium. Attempts to detect proteolytic enzymes in the toxin of Cl. oedematiens have failed [5, 6]. Our repeated attempts to find enzymes in the culture medium at different stages of growth have also given negative results.

It is therefore interesting to consider the question of the presence of proteases in the cells of Cl. oedematiens and to study their properties.

EXPERIMENTAL METHODS

The proteolytic activity was studied in a suspension of Cl. oedematiens Strain No. 794, which was prepared from a 24-hour culture grown on a casein medium and washed free of culture liquid. The microbe mass was ground with powdered glass and diluted with water in the ratio 1 : 10. We added 4 ml of 10% bacterial suspension and 2 ml of 2% casein solution to 10 ml of a phosphate-citrate buffer with pH 7.8. The sample was incubated in a thermostat at 37° for 24 hours. Toluol was used as a preservative. An equal volume of 10% CCl_3COOH was added to the sample to stop the action of the proteolytic enzyme. The occurrence of proteolysis was determined by rise in amino nitrogen which was determined colorimetrically with Nessler reagent. The results were expressed in mg % N_2 per dry weight.

EXPERIMENTAL RESULTS

Since we detected a protease in the cells of Cl. oedematiens we studied the effect of pH on the activity of the enzyme. Fig. 1 shows that the protease has a wide range of activity, with optimum pH at 7.8.

Fig. 2 gives the data on the activity of the protease on various protein substrates (in 2% concentration). In the experiment with gelatin we used 50% CCl_3COOH instead of 10% to inactivate the proteolytic enzyme and precipitate the protein. To measure self proteolysis the sample was allowed to stand without addition of a substrate. We also studied the action of the enzyme on collagen, which was broken down along with the other proteins. The question of the presence in Cl. oedematiens cells of a collagenase which differs from the protease requires further study.

Casein is broken down to a greater degree than any other protein substrate used.

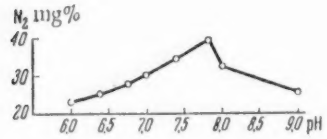

Fig. 1. Effect of pH on protease activity of Cl. oedematiens.

When we studied the action of SH compounds on the protease activity, we found that cysteine, glutathione, and thiolactic acid (taken in equivalent amounts with respect to the SH group, M/400) activated the protease; cysteine had more effect than the other substances on the enzyme activity (Fig. 3).

According to the literature, certain chemical compounds inhibit the action of proteolytic anzymes; monoiodoacetic acid, sodium azide, ascorbic acid, hydroxyquinoline [7], and also native serum [8].

In our experiments we used the following concentrations: monoiodoacetic acid, 0.0013 M; sodium azide, 0.0038 M; ascorbic acid, 0.0014 M; hydroxyquinoline, 0.008 M. We used 20% solutions of these native sera: normal horse serum, specific oedematiens, perfringens, tetanus and diphtheria sera.

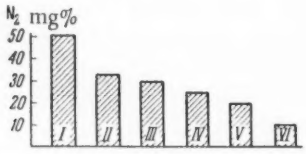

Fig. 2. Action of the protease of Cl. oedematiens on protein substrates.
I) Casein, II) gelatin, III) protein from microbe mass of Cl. oedematiens, IV) egg albumin, V) collagen, VI) self-proteolysis.

Table 4 shows that monoiodoacetic acid, sodium azide, normal horse serum and specific oedematiens serum inhibited the action of the enzyme. Hydroxyquinoline inhibited the protease. Ascorbic acid and the other sera had no effect on enzyme activity.

Weil and Kocholaty [9] concluded from a study of the protease of Cl. histolyticum that metal ions in low concentration activated the enzyme. We also studied the effect of divalent ions on the protease activity.

The experiments were performed at a pH of the medium of 7 instead of 7.8 to avoid precipitation of the salts. Cu^{++}, Co^{++}, Fe^{++}, Ni^{++}, Mg^{++} were studied in concentrations of 0.012 M. For Zn^{++}, Mn^{++}, Ca^{++}, and Cd^{++} 0.006 M solutions were used.

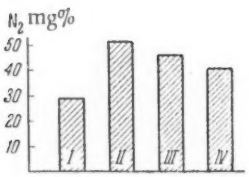

Fig. 3. Effect of SH compounds on the protease activity of Cl. oedematiens.
I) Control, II) cysteine, III) glutathione, IV) thiolactate.

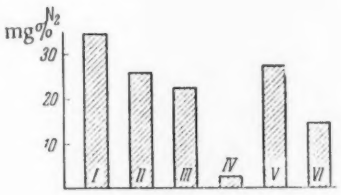

Fig. 4. Effect of inhibitors on the protease activity of Cl. oedematiens
I) Control, II) sodium azide, III) monoiodoacetate, IV) hydroxyquinoline, V) normal horse serum, VI) specific oedematiens serum.

Fig. 5 shows that among these ions only Zn^{++}, Cu^{++}, and Cd^{++} inhibited the protease strongly; the other salts did not change enzyme activity.

SUMMARY

In a study of the proteolytic activity of Cl. oedematiens we found a protease with an optimum pH 7.8 which split casein, gelatin, collagen, egg albumen, and the protein which we isolated from the bacterial cell. The pro-

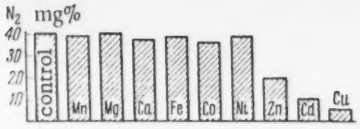

Fig. 5. Effect of metal ions on the protease activity of Cl. oedematiens.

tease was activated by SH compounds. Divalent metal ions had no effect on the proteolytic activity of Cl. oedematiens, with the exception of Zn^{++}, Cu^{++}, and Cd^{++} which inhibited it. Monoiodoacetic acid and sodium azide slightly inhibited this protease. Hydroxyquinoline prevented the action of the enzyme. Among a number of active sera, only normal horse serum and the specific oedematiens serum decreased the activity of Cl. oedematiens protease.

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THE REACTION OF ADENOSINE TRIPHOSPHATE WITH DYES

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It is generally accepted that vital staining by dyes depends on the proteins contained in cells and tissues. This idea is based on the ability of isolated proteins to combine with dyes and also on the greater amounts of proteins compared to other constituents of protoplasm [1, 2]. It is also known, however, that the nonprotein constituents of protoplasm such as polysaccharides, lipids, and nucleic acids have a definite effect on the staining properties of cells [3-5]. The nucleic acids, particularly,are thought to have a primary role in the basophilic reaction of protoplasm [6, 7].

Among the protoplasmic constituents which can react with dyes and which have not previously been studied, adenosine triphosphate (ATP) is especially interesting. ATP is present in the protoplasm of all cells and its concentration in skeletal muscle is almost 0.005 M [8]. The presence of ATP of many kinds of chemical groups, some with definite acid or basic properties, and the ease with which positive or negative charges may occur in the molecule suggest the possibility that it may react with both basic and acid dyes.

PREPARATIONS AND METHODS

In this work we used ATP produced by the Ivanov Meat Combine in 1955. Chromatographic analysis of the preparation showed that it contained ADP and AMP. The content of inorganic P was low (less than 0.5%). A sample of ATP was dissolved in twice-distilled water and neutralized with 0.1 N NaOH to a given pH. The dyes used were methylene blue (Kahlbaum), toluidine blue, Nile blue sulfate (Grubler), neutral red, and Congo red (Kharkov Alkaloid Factory). In these experiments we used commercial preparations of all the dyes except neutral red, which was purified through the anhydro base.

We used a spectrophotometric method in most cases for detection of a reaction between ATP and a dye. In some experiments we also used dialysis of the mixture of ATP and dye through cellophane and diffusion on gelatin.

EXPERIMENTAL DATA

Dialysis of Solutions of ATP and Dyes to Equilibrium

The experiments were carried out as follows: 3 ml of buffered ATP solution was placed in a cellophane bag which was immersed in a beaker containing 10 ml of dye solution ($0.3 \cdot 10^{-3}$ M). The salt concentrations and pH of the inner and outer solutions were equal. In control experiments the bag contained 2 ml of buffered solution but no ATP. Dialysis took place at 4-6° in a refrigerator for 40 hours. Then the amount of dye in the inner and outer liquids was determined, and also the amount adsorbed on the walls of the bag. Determination of the dye was carried out with an FEK-M photometer.

The data for the experiments with dialysis are given in Table 1.

The results gave some indication of complex formation between ATP and dyes. This was especially suggested by the greater rapidity in reaching equilibrium for the dye inside and outside the bag in the experimental samples, and also by the accumulation of dyes within the bag. However, the quantitative evaluation of the results was complicated by a number of factors. As our study showed, ATP and perhaps also its complex with the dyes could diffuse slowly through the cellophane; the tint of the colored solution changed in the presence of ATP; and in the tests with neutral red a colored precipitate formed.

TABLE 1

Effect of Adenosine Triphosphate on Dialysis of Dyes

Dye	Duration of experiment, hours	ATP in mg/ml	Concentration of buffer in M		pH	Sample absorption in E						III/I	IV/III
						Control			With ATP				
			Acetate	Phosphate		Outside I	Inside II	Walls	Outside III	Inside IV	Walls		
Methylene blue	40	2.4	0.05	—	5.0	0.59	0.43	0.17	0.58	0.74	0.14	0.72	1.27
The same	40	1.2	0.1	—	6.0	0.44	0.32	0.13	0.36	0.38	0.12	0.72	1.05
"	41	2.4	—	0.06	7.2	0.42	0.37	0.13	0.39	0.42	0.11	0.88	1.07
"	41	1.2	—	0.06	7.2	0.42	0.37	0.13	0.48	0.44	0.10	0.88	1.02
"	41	0.6	—	0.06	7.2	0.42	0.37	0.13	0.40	0.42	0.12	0.88	1.05
Neutral red	40	2.4	0.1	—	6.2	0.37	0.21	0.32	0.55	0.46	0.24	0.56	0.83

Diffusion of a Mixture of Dye and ATP on Gelatin

The technique of the diffusion experiments on gelatin was the same as has been described [9]. It was easy to see differences in penetration of dye into the gelatin visually: in samples which contained ATP the depth of pentration of dye into the gel was always greater than in the controls, and, what was very characterictic, the dye did not concentrate on the gelatin surface, but penetrated it diffusely (Table 2).

TABLE 2

Distribution of Neutral Red in the Presence of ATP Between a Solution and a Gelatin Gel After Diffusion for 17 hours.

Dye Concentration $0.3 \cdot 10^{-3}$ M

ATP concentration, mg in solution above gelatin	Dye concentration in solution above gelatin, in E	Shade of solution above the gelatin	Dye concentration in gel in E	Type of penetration into gel
2.4	0.45	Crimson	0.33	Diffuse
1.2	0.41	"	0.35	"
0	0.38	Yellowish	0.35	Sharply defined layer on surface

The change in type of diffusion of dye into the gel under the influence of ATP was easily explained as a result of disaggregation of the dye owing to the formation of a complex with ATP. The same effect has been found in studies of the diffusion into gelatin of a mixture of dyes with proteins, alcohol, pyridine, and other hydrotropic substances, and the same explanation has been given [10]. The relatively low ATP concentration which produces this effect is worthy of notice. This indicates the considerable tendency of ATP to form complexes.

Effect of ATP on the Absorption Spectra of Dyes

These results served as the basis for the study of the influence of ATP on the absorption spectra of dye solutions in the visual range.

A mixture of dye, ATP and buffer solutions was prepared for the spectrophotometric measurements with the following composition: 1 ml of aqueous solution of dye (10^{-3} to 10^{-4} M), 0.5 ml of buffer solution (acetate or phosphate, 0.1-0.13 M, pH 5-7), 1 ml of neutralized ATP solution (10^{-3} to 10^{-2} M), and water to 4 ml. In a number of cases during the study, a neutral salt was added. For this purpose we prepared a mixture which contained besides the dye, ATP, and buffer, 0.5 ml of 2 M KCl; in the control tests, ATP was not added. All the measurements were made on an SF-4 spectrophotometer at room temperature.* Dye solutions with concentrations of about 10^{-4} M were studied in a 10 mm cuvette; more concentrated solutions in a 1 mm cuvette.

* We wish to thank A. L. Arvan for help in the spectrophotometric study.

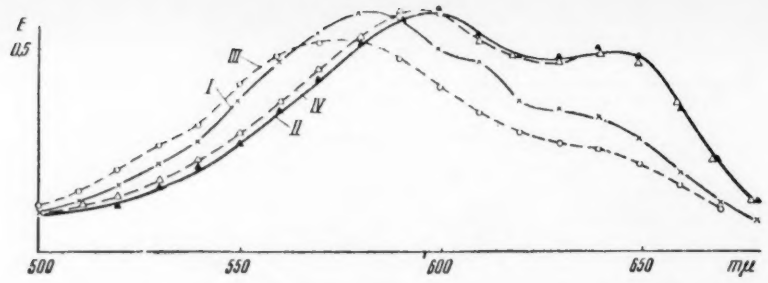

Fig. 1. Effect of ATP on the absorption spectrum of aqueous and salt solutions of toluidine blue (pH 6).
I) Aqueous dye solution $1.17 \cdot 10^{-3}$ M; II) the same with $1.1 \cdot 10^{-2}$ M ATP; III) dye solution in 0.25 M KCl; IV) dye solution in the presence of $1.1 \cdot 10^{-2}$ M ATP and 0.25 M KCl.

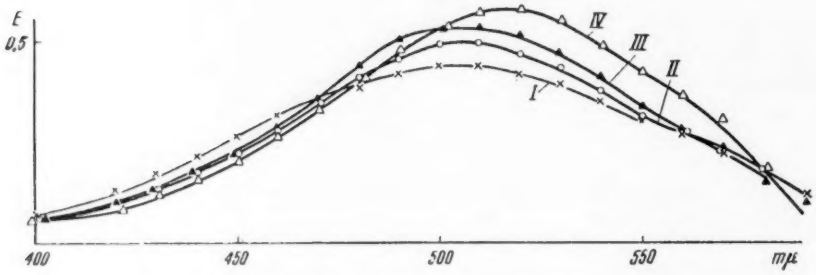

Fig. 2. Effect of ATP on the absorption spectrum of neutral red (pH 6).
I) Dye solution $0.3 \cdot 10^{-3}$ M; II) the same with $0.5 \cdot 10^{-3}$ M ATP; III) the same with $1.1 \cdot 10^{-3}$ M ATP; IV) the same with $1.1 \cdot 10^{-2}$ M ATP.

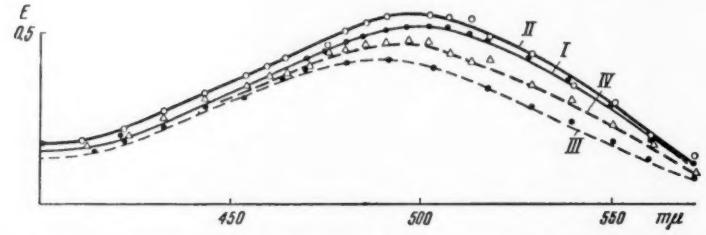

Fig. 3. Effect of ATP on the absorption spectrum of aqueous and salt solutions of Congo red (pH 6).
I) Aqueous dye solution $1.3 \cdot 10^{-4}$ M; II) the same in the presence of $0.5 \cdot 10^{-3}$ M ATP; III) dye solution in 0.25 M KCl; IV) dye solution in 0.25 M KCl with $0.5 \cdot 10^{-3}$ M ATP.

Figs. 1-5 and Table 3 show that when ATP was added to the dye solutions, it caused a distinct increase in intensity of absorption (hyperchromic effect), and also produced a shift in absorption maximum of 5-20 mμ. These effects were observed for the basic dyes toluidine blue, methylene b'ue, Nile blue, and neutral red, and for the acid dye, Congo red. The higher the concentration of ATP, the greater was its effect (Table 3). No particular effect of pH in the range 5-8 was noted for this reaction.

The change in optical properties of the dye solutions under the influence of ATP was particularly well demonstrated when a neutral salt was added. It is known that in the presence of salts the absorption maxima for dyes shifts toward the shorter wave lengths and the absorption intensity decreases [2, 11]. When ATP was added

to salt solutions of toluidine blue or Congo red, the absorption spectra shifted toward the long wave lengths, the absorption intensity increased at the same time (to a greater extent, the higher the ATP concentration) (Fig. 7).

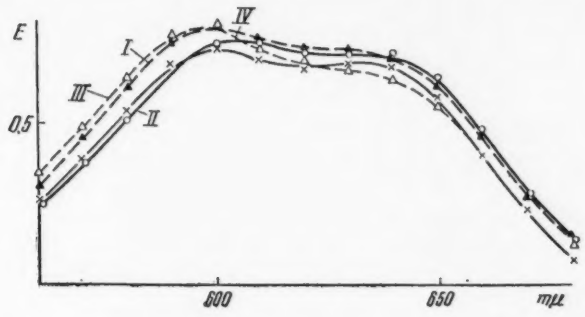

Fig. 4. Effect of ATP on the absorption spectrum of aqueous and salt solutions of Nile blue sulfate (pH 6).
I) Aqueous dye solution $4.9 \cdot 10^{-4}$ M; II) the same with $0.5 \cdot 10^{-3}$ M ATP; III) dye solution in 0.25 M KCl with $0.5 \cdot 10^{-3}$ M ATP.

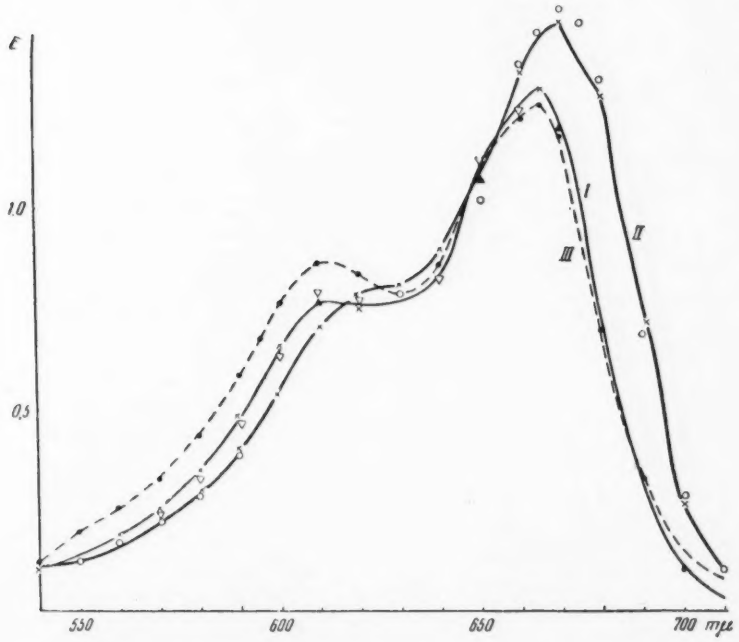

Fig. 5. Effect of ATP on the absorption spectrum of aqueous and salt solutions of methylene blue (pH 6).
I) Aqueous dye solution 10^{-4} M; II) the same in the presence of $1.1 \cdot 10^{-3}$ M ATP; III) dye solution in 0.25 M KCl.

The effect of ATP on the absorption spectra of dyes is best shown graphically. To do this we used the method of Kravets, who showed that the surface contour of the spectrum curve and also the sum of the optical densities in a given section of the curve could characterize the changes which occurred in the spectrum properties of the solution [13]. In Fig. 6 we give the results of calculations of the sum of optical densities of toluidine blue and Congo red for the section of the curve from 500 to 680 mμ for the first (Fig. 1), and from 400 to 560 mμ (Fig. 3) for the second dye.

TABLE 3

Effect of ATP on the Absorption Spectra of Dyes

Dye	Final dye concent. in M	Absorption max. in mμ	Shift of max. to right in mμ		
			Concentration of ATP in M		
			$0.5 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$	$1.1 \cdot 10^{-2}$
Neutral red	$0.3 \cdot 10^{-3}$	500	0	5	17
Toluidine blue	$0.39 \cdot 10^{-4}$	630	10	—	—
	$1.17 \cdot 10^{-3}$	580	—	10	20
Methylene blue	$0.3 \cdot 10^{-3}$	665	—	—	5

Figure 6 shows that the addition of ATP to salt solutions of toluidine blue and Congo red increased the surface contour by 17 and 15%.

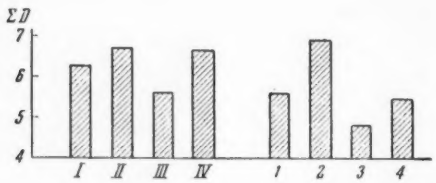

Fig. 6. Sum of the optical densities for toluidine blue at 500-680 mμ and for Congo red at 400-560 mμ.
I) Aqueous solution of toluidine blue $1.17 \cdot 10^{-3}$ M; II) the same in the presence of $1.1 \cdot 10^{-2}$ M ATP; III) solution of toluidine blue in 0.25 M KCl; IV) solution of toluidine blue in 0.25 M KCl with $1.1 \cdot 10^{-2}$ M ATP.
1) Solution of Congo red $1.3 \cdot 10^{-4}$ M; 2) the same with addition of $1.1 \cdot 10^{-2}$ M ATP; 3) solution of Congo red in 0.25 M KCl; 4) solution of Congo red in 0.25 M KCl with $1.1 \cdot 10^{-2}$ M ATP.

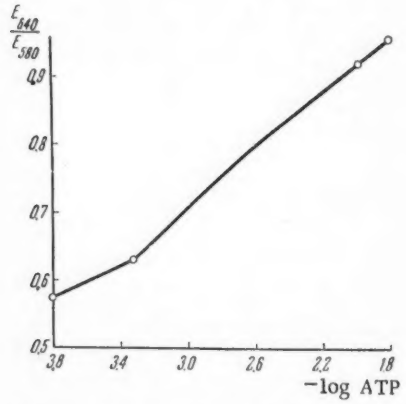

Fig. 7. Change in curve of spectrum of toluidine blue ($1.17 \cdot 10^{-3}$ M) under the influence of ATP.

Another good indicator of changes in optical properties of dye solutions is the ratio of long wave to short wave maxima [11].

Fig. 7 gives these values, calculated from the data of Fig. 1. The curve shows that as the concentration of ATP increased, there was a marked decrease in concentration of the aggregated form of the dye.

DISCUSSION OF RESULTS

It is known that in aqueous solution the dyes are aggregated. For some dyes two molecules are aggregated (dimer), for others there are larger aggregations (trimers, polymers). Aggregation of dyes increases sharply with their concentration and also when neutral salts are added. This produces anomalous properties in the dye solutions examples of which are: characteristic changes in the absorption spectra of the solutions in the visual field: the shift of the absorption maxima to shorter wave lengths and a general decrease in intensity of absorption. It is still uncertain how the aggregation of particles is maintained, but most authors tend to believe that an important factor is the molecular binding force (van der Waals force).

The nature of the changes in spectrum properties of these dyes under the influence of ATP shows that disaggregation occurs. Similar effects on the state of dyes in solution have been observed when proteins, alcohol, pyridine, and other substances are added to them. The mechanism of this effect has been thoroughly studied and explained by the formation of complexes between the dye and the substances which are added [11]. Therefore we are justified in concluding that the disaggregation of the dyes produced by ATP is also related to formation of a compound of ATP and dye. This conclusion agrees with the results of the experiments on dialysis of mixtures of ATP and dye through cellophane and diffusion into gelatin.

The conclusion as to the disaggregation of dyes by

ATP also agrees with the data of Mandl and Neuberg [12] who observed high solubilizing activity for ATP. They give many examples of reaction of ATP with difficultly soluble substances; as a result of such reactions the complex becomes soluble so that it can penetrate into the cell and be transferred within the cell.

This ability of ATP to combine with acid and basic dyes can certainly be an important factor in the staining of cells and tissues in various functional conditions.

According to Nasonov and Aleksandrov [1] processes of stimulation or injury to protoplasm are related to alterations in proteins similar to those in the initial stages of denaturation. One of the consequences of such changes in the proteins is the weakening and suppression of the attraction to a number of smaller constituents of protoplasm with which the proteins combine in the resting and uninjured cell. Compounds of proteins with ATP, ADP, creatine, creatine phosphate, nucleic acids, etc. are decomposed, and some of these substances may diffuse from the cell into the surrounding fluids. Another results is the liberation of many functional groups of protein and nonprotein components and this leads to a general increase in nonspecific chemical activity of the living substrates. The effects appear as increased activity of intracellular proteins and intermolecular reactions, changes in the colloidal state of the proteins, their increased attraction for dyes, etc.

We can conclude form our data that the nonprotein components share with the proteins a definite state of adsorption in protoplasm, and that their union with proteins varies with the physiological condition of the cell.

SUMMARY

Adenosine triphosphate (ATP) changes the nature of the diffusion of basic and acidic dyes through cellophane and into gelatin and also affects the properties of the spectra of dye solutions in the visual field. The data suggest disaggregation of the dyes by the action of ATP owing to formation of a complex of ATP with the dyes. The significance of these results for the staining reactions of protoplasm is discussed.

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THE PROBLEM OF DETERMINATION OF GLYCOGEN IN THE BLOOD

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The method of Pflüger is used for determination of glycogen in tissues; it consists in the alkaline hydrolysis of the tissue, and precipitation of the glycogen from the alkaline hydrolyzate by alcohol. The method can be used in its original form or with variations. The most widely used modification is that of Good, Kramer, and Somogyi [1], in which the alcoholic precipitation is carried out with heating to hasten the process.

Until recently the existence of two forms of glycogen was recognized: "free," which was extracted by weak trichloroacetic acid solution, and "bound," which required alkaline hydrolysis for extraction. However, in recent years it has been shown that there is no essential difference between these two glycogen fractions. In 1947 and 1948 Rozenfel'd [2, 3] and later Roe and his co-workers [4] showed that repeated extraction with trichloroacetic acid solutions removed as much glycogen as was obtained after alkaline hydrolysis. At the same time Roe and co-workers found that when alcoholic precipitation was used, some kind of low-molecular weight (dialyzable) polysaccharide precipitated with the glycogen; its nature was not explained.

In the present work we describe some new facts which give a closer approach to the solution of the problem of the nature of blood polysaccharides and methods for determining them.

The original purpose of the work was to improve the Pflüger method. The alkaline hydrolyzate was to be neutralized and the impurities precipitated by trichloroacetic acid; then a single alcoholic precipitation would give pure glycogen. However, the first experiment gave an unexpected result: the amount of glycogen after neutralization was twice as great as when it was precipitated from the alkaline hydrolyzate. It was necessary to explain this difference.

METHODS

One ml of blood was added to 2 ml of 30% KOH and the mixture was hydrolyzed for 20 minutes on a boiling water bath. After cooling, it was treated with 2 ml of 26% H_2SO_4, again cooled, and 1 ml of 30% trichloroacetic acid was added. When this was centrifuged, it gave a clear, slightly opalescent, pale yellow liquid. For precipitation 1 ml of the centrifugate was treated with 5 ml of 96% alcohol. This caused precipitation with the glycogen of a voluminous precipitate of salts which adhered strongly to the test tube and prevented loss of glycogen when the centrifugate was poured off. A single precipitation gave sufficiently pure glycogen; repeated precipitations did not change the results of the determination. Results of a determination in which 500 μg of glycogen was added to the sample were:

	Without adding glycogen (μg)	With addition of glycogen (μg)
Neutralized, purified with trichloroacetic acid, one precipitation	530	1130
Neutralized, purified with trichloroacetic acid, two precipitations	580	1090

The next day the precipitate was separated by centrifuging, mixed with 2 ml of 80% alcohol, and again centrifuged. The precipitate was dissolved in 2 ml of water, 1 ml of this was transferred to a test tube, treated with 5 ml

of 0.05% solution of anthrone in 72% H_2SO_4 and heated on a boiling water bath for exactly 15 minutes [5]. The color which developed was measured on an FEK-M photoelectric colorimeter.

A standard curve was constructed by using a solution of glucose in saturated benzoic acid [5].

For purposes of comparison, glycogen in the precipitate was determined in some cases by a reductometric method (HCl hydrolysis and determination of reducing substances by the Hagedom-Jensen method).

RESULTS

Table 1 gives the amount of glycogen found in human blood by precipitation with alcohol from the alkaline hydrolyzate and from the hydrolyzate after neutralization and purification with trichloroacetic acid.

TABLE 1

Amount of Glycogen in Human Blood under Different Conditions of Precipitation and Different Methods of Determination (glycogen in μg in sample)

Anthrone method		Reductometric method		Anthrone method		Reductometric method	
Pption. from alkaline med.	Pption. after neutralization	Pption. from alkaline medium	Pption after neutralization	Pption. from alkaline med.	Pption. after neutralization	Pption. from alkaline medium	Pption. after neutralization
120	360	—	—	40	230	—	—
290	520	—	—	100	300	—	—
100	410	—	—	40	200	—	—
110	800	—	—	40	160	—	—
180	450	310	1600	60	310	—	—
280	520	620	1520	80	300	—	—
170	410	—	—	100	530	290	1120
140	340	330	1400	150	530	120	860
230	300	360	1070	200	730	—	—
330	500	—	—	160	580	—	—
200	540	490	630	220	490	—	—
350	1040	530	720	170	680	130	1320
180	270	380	1410	90	380	—	—
120	450	230	1370				
310	450	—	—				
120	430	140	960	Average	432±11	325±44	1139±82
80	230	490	1100	153±15 (*I*)	(*II*)	(*III*)	(*IV*)
80	160	130	860	(p=0.10)	(p=0.02)	(p=0.13)	(p=0.07)
50	220	—	—	difference *II*—*I* is significant (t=15)		difference is significant	*IV*—*III* (t=8.95

The glycogen from the alkaline hydrolyzate was reprecipitated three times; glycogen from the fraction which was neutralized but not purified by trichloroacetic acid was twice precipitated, and after purification with trichloroacetic acid, one precipitation was enough.

Table 1 shows that after precipitation of glycogen from the neutralized hydrolyzate, its amount in the blood, determined by the anthrone method, was five times higher than when it was precipitated from the alkaline medium (samples neutralized without purification by trichloroacetic acid gave the same results as when they were purified with trichloroacetic acid). Analogous results were obtained in several experiments with lung tissue (obtained in operations). The same results were obtained in determinations of glycogen by the reductometric method, though the absolute values obtained by the anthrone and reductometric methods were quite different (Table 1).

The results of these experiments did not permit an explanation of the increase in amount of glycogen when the alkaline hydrolyzate was neutralized. The data from the reductometric determination of glycogen made it seem improbable that the formation of large amounts of salt during neutralization affected the color of the anthrone reaction. Experiments in which the standard glucose solution was treated with the same amount of KOH as in the determination of glycogen in blood, and then with neutralization by sulfuric, hydrochloric, or acetic acid, showed that sulfate did not affect the intensity of the color. Any idea of the influence of salts was also contradicted by the data of Table 2.

TABLE 2

Determination of Glycogen in Water Solution

(glycogen in μg in sample)

Experiment No.	Solution of glycogen + + anthrone reagent (without ppt. by alcohol) I	Precipitation by alcohol		
		Glycogen solution without additions II	Glycogen solution + KOH III	Glycogen solution + KOH, neutralized IV
1	1000	910	920	960
2	460	300	400	400
3	570	400	340	430
4	490	390	430	480
5	390	—	320	410

Note: In experiment 1, 1 mg of glycogen was used in the sample, in the other experiments, 500 μg.

We could assume that in an alkaline medium glycogen was not completely precipitated, or that in a neutral medium alcohol precipitated some sort of polysaccharide which differed from glycogen and was not precipitated in an alkaline medium.

Table 2 gives data from experiments with a water solution of glycogen. The results of determination in alkali and neutral media were similar.

The difference between the values of columns IV and III was not significant ($t < 0.5$). The much lower values in some of the samples of columns II and III as compared to column I were due to the fact that when glycogen was precipitated from solutions which did not contain salt, the precipitate was not voluminous and adhered poorly to the bottom of the tube; when the centrifugate was poured off, part of the precipitate could have been lost.

It would seem that the idea of incomplete precipitation of glycogen was not supported. However, the results of the experiments on adding glycogen to the alkaline hydrolyzate from blood, given in Table 3, showed that the blood glycogen was not completely determined when alcoholic precipitation took place in an alkaline medium. Analogous results were obtained when dextran was added to blood (the preparations of glycogen and dextran were kindly supplied by E. L. Rozenfel'd).

TABLE 3

Determination of Glycogen and Dextran Added to Blood

To 1 ml was added 500 μg of glycogen or dextran (glycogen or dextran in μg in sample)

Precipitated from alkaline medium					Precipitated after neutralization				
Blood without add.	Blood + glycogen		Blood + dextran		Blood without add.	Blood + glycogen		Blood + dextran	
	Calc.	Found	Calc.	Found		Calc.	Found	Calc.	Found
180	680	250	—	—	450	950	1080	—	—
200	700	550	—	—	540	1040	1140	—	—
350	850	800	—	—	1040	1540	1620	—	—
120	620	320	—	—	450	950	1090	—	—
100	600	290	—	—	530	1030	1120	—	—
150	650	150	—	—	530	1030	1130	—	—
170	670	180	670	300	680	1180	1250	1180	1250
120	620	180	620	150	430	930	870	930	900
80	580	170	580	90	230	730	650	730	730
80	580	120	580	130	160	660	570	660	620

When precipitation took place in a neutral medium, added glycogen and dextran were determined completely, but when precipitation occurred in an alkaline medium, only 12-30% was found.

The data of Table 2 showed that in itself the reaction medium had no particular significance. This suggested that in the alkaline hydrolyzate of blood there were substances which combined with glycogen and prevented its precipitation. To test this idea we centrifuged the blood hydrolyzate after its neutralization (before adding trichloroacetic acid), dissolved the precipitate in 30% KOH, and added to this solution glycogen (or water) and then precipitated the glycogen by alcohol and determined it quantitatively.

The results of these experiments, given in Fig. 1, showed that substances which hindered the precipitation of glycogen by alcohol actually existed and that they precipitated during neutralization of the alkaline hydrolyzate.

Yet if glycogen was not fully precipitated from the alkaline hydrolyzate, it should remain in the solution.

The centrifugate after precipitation of glycogen from the alkaline hydrolyzate was poured off separately, the alcohol was evaporated, and the aqueous residue was neutralized and purified by trichloroacetic acid as previously described. However, as Table 4 shows, glycogen could be determined in the centrifugate in only one case.

TABLE 4

Glycogen Remaining in Solution after Precipitation from an Alkaline Medium

(glycogen in μg in sample)

Precipitated from alkaline medium	Precipitated after neutralization	Glycogen after pption. from alkaline medium
90	380	300
40	230	0
100	300	50
0	350	0
40	200	10
40	160	0

TABLE 5

Amount of Glycogen at Different Times of Hydrolysis

(glycogen in μg in sample)

Ppt'd from alkaline medium		Ppt'd after neutralization	
Hydrolysis, 20 min	Hydrolysis, 2 hours	Hydrolysis, 20 min	Hydrolysis, 2 hours
200	250	730	530
160	240	580	440
220	230	490	500

In a recently published paper Roe and co-workers [4] have expressed the opinion that during alkaline hydrolysis of the tissues, low-molecular weight, dialyzing polysaccharides are liberated; the authors call them "non-glycogenic," but they precipitate with the glycogen. According to the data of these authors, these polysaccharides differ from glycogen in that they are decomposed by long heating with strong alkali. Table 5 shows the results obtained using different periods of alkaline hydrolysis. In two of the three experiments the amount of glycogen after 2-hour hydrolysis was somewhat less than after 20-minute hydrolysis.

The blood hydrolyzate, neutralized and purified with trichloroacetic acid, was tested by dialysis through cellophane against 3 volumes of distilled water for 18-20 hours (in one experiment, for 42 hours). In most experiments there was a decrease in polysaccharide inside the bag, but only in special cases were polysaccharides found in the outside solution.

DISCUSSION

These results show that when glycogen is precipitated by alcohol from an alkaline medium it does not precipitate completely, but glycogen can be determined in the centrifugate only in individual cases.

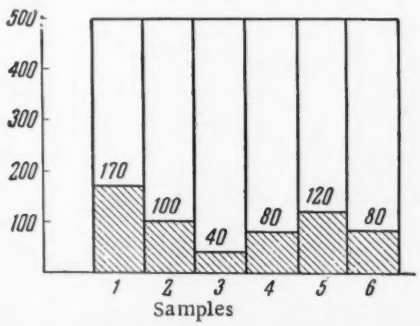

Fig. 1. Effect of substances which precipitate during neutralization of alkaline blood hydrolyzate on determination of glycogen.

To each sample was added 500 μg of glycogen (total height of column). The shaded part of the column is the analytically determined amount of glycogen.

Table 4 shows that there are substances in the alkaline hydrolyzate from blood (and possibly from tissue) which hinder the precipitation of glycogen by alcohol. These substances precipitate during neutralization of the alkaline hydrolyzate. The nature of these substances requires further study, but we can assume that they are decomposition products of proteins.

Genkin [6] showed that glycogen extracted from the liver by physiological saline was almost completely bound to protein and could be separated from it by acidification of the solution. The same author showed [7] that combination of glycogen and protein could occur in solution and also that treatment of glycogen with alkali did not destroy completely its ability to form complexes with proteins.

When the neutralized hydrolyzate is dialyzed it is possible to find a decrease in the amount of polysaccharides in the bag, but in most cases polysaccharides

are not detected in the outside solution. This fact is still unexplained.

Two-hour alkaline hydrolysis results in some decrease in quantity of polysaccharides, but this is not found in all experiments. Thus the opinion of Roe and co-workers [4], that after alkaline hydrolysis some sort of low molecular weight polysaccharide which decomposes when boiled with alkali is determined with the glycogen, is not fully confirmed by our work, though it cannot be rejected on the basis of our data. Therefore, although in this paper we say for simplicity "glycogen," we actually refer to the polysaccharides of the blood.

Our results for the glycogen content of human blood determined by precipitation from an alkaline medium (Table 1) agree with the literature data [8, 9] for the average value (15.3 mg%) and the range of variations. The higher values obtained by the reductometric method (Table 1) can be explained by the presence in the glycogen precipitate of other reducing substances. Genkin [10] and Shatalova [9] have mentioned the existence of such impurities. It is possible that the higher values obtained by the Japanese authors [11] are also the result of reducing impurities.

SUMMARY

When blood glycogen is determined by the Pflüger method, neutralization of the alkaline hydrolyzate before alcoholic precipitation of the glycogen greatly changes the results of the determination; the amount of glycogen in this case is two to five times higher than when it is precipitated from the alkaline hydrolyzate. The same results are obtained in determining the glycogen content of lung tissue.

The determination of glycogen in a water solution gives the same results in an alkaline or neutral medium. Glycogen added to the alkaline hydrolyzate of blood and precipitated by alcohol from the alkaline medium is not determined completely; neutralization of this hydrolyzate assures full determination of the added glycogen. The same results are obtained when dextran is added to the blood.

The precipitate obtained with neutralizing the alkaline blood hydrolyzate, when dissolved in KOH, hinders full precipitation of any glycogen added to it.

After 2-hour alkaline hydrolysis of blood the amount of glycogen in the neutralized hydrolyzate (in 2 out of 3 experiments) is somewhat less than after 20-minute hydrolysis.

When the neutralized hydrolyzate is dialyzed, in most experiments there is a decreased amount of polysaccharide inside the bag, but only in special cases can polysaccharides be detected in the outside liquid.

In conclusion, I consider it my duty to express my thanks to E. L. Rozenfel'd, Doctor of Biological Science, for his valuable advice and help in this work.

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A STUDY OF THE N-TERMINAL GROUPS IN THE ZEIN OF "DAGESTAN" CORN

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The best method for determining the N-terminal groups of proteins is through the dinitrophenyl derivatives of amino acids (DNP-amino acids) of Sanger [1]. By 1954 the terminal groups of 57 proteins had been determined by use of the Sanger method [2]. The plant proteins have been little studied, however. It is known that in zein the N-terminal group contains threonine and glutamic acid [3].

EXPERIMENTAL

For this study we synthesized 20 DNP-amino acid derivatives: glycine, alanine, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, aspartic and glutamic acids, methionine, cystine, lysine (bis-DNP--lysine and mono-DNP-lysine), arginine, histidine (bis-DNP-histidine and mono-DNP-histidine), tryptophan, and proline.

Chromatographic analysis was carried out in three different solvents: aqueous solution of sodium citrate (pH 6), phenol saturated with water, and "toluene" used by Biserte and Osteux [4] with a change in the ratio of components (toluene-pyridine-ethylenechlorohydrin-0.8 N ammonia in the ratio 1 : 1 : 0.1 : 0.1 by volume).

Since the R_f values of DNP-amino acids vary greatly with a number of factors [4], the absolute significance of these values is not very characteristic of the compounds. However, in every individual solvent several groups of DNP-amino acids can be separated since they move at different rates. Thus, the 18 DNP-amino acids which we studied in chromatograms using citrate with pH 6 in orienting experiments could be divided into three groups on the basis of R_f values. The first group of slowly moving DNP-amino acids contained DNP-tyrosine, bis-DNP--lysine, bis-DNP-histidine, and the by-product, dinitroaniline. The second and largest group contained the DNP--derivatives of glycine, cystine, methionine, phenylalanine, arginine, leucine, isoleucine, alanine, serine, proline, valine, threonine, and mono-DNP-lysine. The third groups moved fastest in citrate and contained the DNP-derivatives of the dicarboxylic acids, DNP-aspartic and DNP-glutamic acids.

It should be noted that DNP-methionine gave two spots, which was not particularly unexpected, since methionine itself gives two spots in chromatographic analysis. The DNP-derivatives of lysine, leucine, proline, aspartic, and glutamic acids also gave two spots on the chromatogram. However, by chromatographic study in three different solvents, treatment of the yellow spots of DNP-amino acid derivatives with hydrochloric acid [5], and conversion of DNP-leucine into leucine by the method of Lowther [6] we were able to show that one of the spots from these DNP-amino acids depended on the presence of dinitrophenol which was formed in the mixture by hydrolysis of dinitrofluorobenzene.

When the chromatogram was prepared with aqueous phenol instead of sodium citrate solution, the spots for aspartic and glutamic acids moved very slowly and made up the first group. Then came a large group of spots for the DNP-derivatives of glycine, serine, alanine, threonine, valine, histidine, proline, phenylalanine, and isoleucine. The R_f values for this group of DNP-amino acids were very similar, so that it was difficult even to establish the order in which they were distributed, since the experimental errors were greater than the differences in their R_f values. The dinitrophenol spot was found in this group. The third group consisted of mono-DNP-lysine, arginine, and dinitroaniline whose spot moved rapidly in phenol and had, naturally, a larger R_f value.

When we used the "toluene" solvent, the DNP-amino acids could be seprated on the basis of their rate of

movement into approximately four groups. The first group of very slow DNP-amino acids consisted of the DNP--derivatives of aspartic and glutamic acids, arginine, and mono-DNP-lysine. The second group (R_f less than 0.5) contained the DNP-derivatives of cystine, serine, glycine, and threonine. The third group contained the DNP-derivatives of alanine, proline, methionine, phenylalanine, lysine, valine, leucine, and isoleucine. The fourth group contained the rapidly moving DNP-derivatives of histidine and tyrosine, and also dinitroaniline.

Determination of the Terminal Groups of Zein

For this work we used zein extracted from "Dagestan" corn from the harvest of 1953, obtained from the Dagestan Experimental Station. DNP-zein was prepared directly [1]. The starting zein contained (in %): N 12.81, 12.94; S 0.38, 0.38; ash 1.1, 1.2. The DNP-zein was hydrolyzed by acid; in different experiments we varied the amount of acid used and the length of heating. Excess hydrochloric acid was removed and the products of hydrolysis were extracted with ether; the ether-soluble DNP-amino acid derivatives were submitted to chromatographic analysis in the three solvents and it was established that the same DNP-derivatives were obtained, regardless of the conditions of hydrolysis.

TABLE 1

R_f Values for DNP-Amino Acid Derivatives from Zein
Solvent – Water Solution of Sodium Citrate (pH 6.0)

Distance of moving front in mm	Spots from hydrolyzate of DNP-zein				DNP-derivatives					
	I	II	III	IV	Histidine	Threonine	Aspartic and glutamic acids	Phenylalanine	Dinitroaniline	Dinitrophenol
88–94	0.345	0.490	0.702	0.810						
88–94	0.358	0.495	0.690	0.798						
88–94	0.300	0.475	0.690	0.828						
88–94	0.338	0.480	0.680	0.810	0.409	0.690	0.810	0.630	0.370	0.495
280–286	0.468	0.642	0.710	0.809	0.432	0.700	0.820		0.340	0.495
280–286	0.470	0.625	0.702	0.820	0.454	0.702	0.820		0.420	0.660
280–286	0.450	0.614	0.720	0.830	0.432	0.690	0.830		0.450	0.640
277–283	0.470	0.626	0.720	0.850						
277–283	0.456	0.620	0.720	0.820						
277–283	0.462	0.606	0.720	0.800						
Average	0.411	0.567	0.702	0.818	0.432	0.697	0.820	0.630	0.395	0.570

Note: The values enclosed in squares show the R_f for the spots in Fig. 1.

Chromatogram of the Hydrolyzate of DNP-Zein Obtained with Sodium Citrate Solution (pH 6.0)

We ran four series of experiments (72 experiments) for the identification of the spots which we obtained. The results are given in Table 1. Figure 1 shows a picture of the movement of the spots.

As Table 1 shows, the ether fraction of the DNP-zein hydrolyzate gives four spots on the chromatogram. The parasitic spot for dinitroaniline (R_f = 0.4) and the spot for bis-DNP-histidine (R_f = 0.432) move along with spot I (R_f = 0.411). It should be noted, however, that when the acid hydrolyzate is extracted with ether, the bis-DNP--histidine should remain in the water fraction [1], in which we did not detect bis-DNP-histidine. Spot II of the DNP-zein hydrolyzate (R_f = 0.567) comes from dinitrophenol (R_f = 0.570). Spot III (R_f = 0.702) moves close to the spot of DNP-threonine (R_f = 0.697), and spot IV of DNP-zein (R_f = 0.818) corresponds to the spot of the DNP--derivatives of glutamic and aspartic acids (R_f = 0.820) which are not separated in this solvent.

According to the data of Deutsch [3], the N-terminal groups of zein are the amino acids phenylalanine and threonine. DNP-phenylalanine was not found by us in the zein hydrolyzate which we studied. Perhaps the presence of dinitrophenol prevented a clear separation of this spot.

Chromatogram of the DNP-Zein Hydrolyzate Obtained with Phenol Saturated with Water

We ran five series of experiments (109 experiments) for identification of the spots from DNP-zein hydrolyzate in the second solvent. The results are given in Table 2.

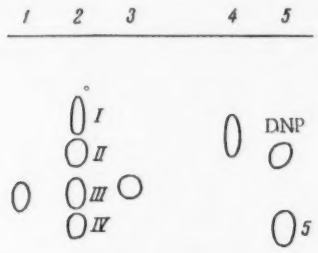

Fig. 1. Scheme of the chromatogram of the ether-soluble part of the DNP--zein hydrolyzate (solvent – sodium citrate, pH 6).
DNP-derivatives: 1) threonine; 2) zein; 3) phenylalanine; 4) histidine ; 5) aspartic and glutamic acids.

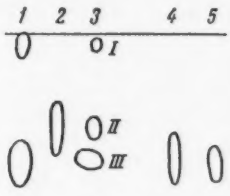

Fig. 2. Scheme of the chromatogram of the ether fraction of the DNP-zein hydrolyzate (solvent – "toluene"). DNP-derivatives: 1) aspartic and glutamic acids; 2) threonine; 3) zein; 4) histidine; 5) dinitrophenol. Under the number 1 the second spot from the top is DNP.

TABLE 2

R_f Values of the Amino Acids of Zein
Solvent – Phenol, Saturated with Water

Distance of moving front in mm	Spots from hydrolyzate of DNP--zein					DNP-derivatives			Dinitrophenol	Dinitroaniline
	I	II	III	IV	V	Aspartic acid	Glutamic acid	Threonine		
167—172	0.225	0.316	0.400	0.572	0.910					
167—172	0.233	0.324	0.415	0.562	0.910					
167—172	0.245	0.350	0.478	0.580	0.910					
167—172	0.283	0.386	0.478	0.648	0.925					
167—172	0.282	0.386	0.480	0.632	0.925		0.380	0.602	0.436	
167—172	0.260	0.345	0.443	0.598	0.917		0.360	0.602	0.448	
124—132	0.366	0.420	0.535	0.635	0.930	0.255	0.380	0.620	0.460	
124—132	0.362	0.427	0.545	0.650	0.925		0.400	0.600	0.549	
124—132	0.350	0.438	0.535	0.645	0.900					
124—132	0.340	0.409	0.500	0.610	0.890					
124—132	0.295	0.350	0.460	0.590	0.850					
124—132	0.295	0.360	0.465	0.610	0.870					0.9—0.95
Average	0.295	0.383	0,475	0.611	0.905	0.255	0.380	0.612	0.470	

As Table 2 shows, the DNP-zein hydrolyzate separates in aqueous phenol into five spots. Spots I and II are very close (R_f = 0.29-0.38) and do not separate in every case. From the R_f values these spots correspond to the DNP-derivatives of aspartic and glutamic acids. Spot III of the DNP-zein hydrolyzate (R_f = 0.475) corresponds to the parasitic spot of dinitrophenol (R_f = 0.470). Spot IV (R_f = 0.61) moves close to DNP-threonine (R_f = 0.61). Spot V of the DNP-zein hydrolyzate moves close to the front of the solvent and so cannot be identified.

Chromatogram of the DNP-Zein Hydrolyzate Obtained with "Toluene"

We ran two series of experiments (26 experiments). The results of the second series are given in Table 3 and Fig. 2.

As Table 3 shows, the hydrolyzate of DNP-zein gives three clear spots. Spot I (R_f = 0.02) corresponds to

DNP-aspartic and DNP-glutamic acids, which are not separated in this solvent; spot II (R_f = 0.22) corresponds to the spot of DNP-threonine (R_f = 0.23); spot III (R_f = 0.32) belongs to dinitrophenol (R_f = 0.31).

TABLE 3

R_f Values of the Amino Acids of Zein
Solvent – "Toluene"

Distance of moving front in mm	Spots from hydrolyzate of DNP-zein			DNP-derivatives		Dinitrophenol	Dinitroaniline
	I	*II*	*III*	Glutamic acid	Threonine		
228	0.026	0.228	0.302	0.022	0.228	0.309	
200—201	0.030	0.236	0.292	0.022	0.235	0.273	
200—201	0.025	0.202	0.310	0.009	0.234	0.317	
178—180	0.013	0.202	0.302	0.032	0.225	0.357	
178—180	0.014	0.217	0.370				
217—218	0.022	0.270	0.350				
217—218	0.016	0.231	0.355				
217—218	0.016	0.231	0.342				
220—221	0.022	0.251	0.364				
220—221	0.014	0.202	0.302				
Average	0.020	0.226	0.329	0.021	0.230	0.314	0.95—1.0

When we summarize the data obtained by chromatography of DNP-zein in three entirely different types of solvent, we can conclude that zein from "Dagestan" corn contains threonine, aspartic, and glutamic acids as N--terminal groups.

SUMMARY

For determination of the N-terminal groups of zein extracted from "Dagestan" corn we prepared the dinitrophenol derivatives of the amino acids and ran a chromatographic analysis of the substances which we obtained.

We found that the N-terminal groups of zein are threonine, aspartic, and glutamic acids.

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THE INCORPORATION OF GLYCINE-1-C^{14} IN THE NUCLEAR PROTEINS OF ANIMAL CELLS WITH REFERENCE TO SOME EFFECTS ON THE DESOXYRIBONUCLEIC ACID OF THE NUCLEUS

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In recent years investigations have shown that both the ribonucleic acid (RNA) of cells and the desoxyribonucleic acid (DNA) of cell nuclei participate in the synthesis of proteins.

Thus, Allfrey, Mirsky, and Osawa showed that after treatment of cell nuclei from calf thymus with desoxyribonuclease (DNA-ase), the inclusion of radioactive amino acids in the nuclear proteins actually decreased; but if these nuclei were then incubated with homologous DNA, the inclusion of labeled amino acids in nuclear proteins rose almost to the original level [1].

The ability of DNA to share in protein synthesis is of particular interest since there is now much evidence to indicate that DNA plays a part in the transmission of hereditary characteristics [2-5]; at the same time the possibility of recording the role of DNA in protein synthesis has independent significance, since it makes it possible to follow the physiological activity of DNA, at least in this relationship.

In the present work we have studied the possibility of restoring the processes of protein synthesis in nuclei which have been treated with DNA-ase or x-rays by incubating them with DNA from the thymus glands of various kinds of animals.

METHODS

Nuclei were separated from the thymus glands of calves and puppies by the sucrose method of Mirsky and Stern [6]. The thymus glands were carefully cut in pieces with scissors, covered with an equal amount of 0.5 M sucrose solution containing 0.002 M $CaCl_2$, and eight times the amount of 0.25 M sucrose solution containing 0.002 M $CaCl_2$ was added. The tissues were homogenized in this solution for 5 min , the suspension was filtered through a double layer of gauze and then a double layer of linen. The filtrate was centrifuged at 600 g for 10 min. The precipitate was suspended in four times its amount of 0.2 M sucrose solution containing 0.002 M $CaCl_2$ and was again centrifuged at 600 g for 10 min. The precipitate of nuclei was again suspended in an equal amount of 0.25 M sucrose containing 0.002 M $CaCl_2$, so that 1 ml of suspension contained 200 mg of nuclei.

When the suspension was viewed under the microscope, the nuclei had the proper round form without cells or cell fragments.

The intensity of protein formation was estimated by inclusion of glycine-1-C^{14} in the nuclear proteins. To a test tube which contained 1 ml of suspended nuclei in 0.25 M sucrose with 0.002 M $CaCl_2$ (200 mg of nuclei) we added 0.5 ml of 0.1 M sodium phosphate buffer in 0.25 M sucrose (pH 7.3) and 0.4 ml of 0.1 M glucose which contained 8.16 mg NaCl and 4.19 mg $MgCl_2$ per 1 ml. We added to this mixture the desired amount of substance in whose influence we were interested (DNA or its hydrolyzate, desoxyribonuclease); after an interval of time which varied with the experimental conditions, we added glycine-1-C^{14} (0.1 ml of solution which contained 8 μC per ml).

The suspension was incubated with glycine-1-C^{14} in a thermostat at 37° for 60 min. At the end of the incubation of the protein, the nuclei were precipitated by adding an equal volume of 10% trichloroacetic acid, the nucleic acids were removed by heating with 5% trichloroacetic acid for 15 min at 90°, and the procedure was repeated. Lipids were extracted from the precipitated protein by boiling in 30-40 times the volume of a mixture of alcohol and ether (3 : 1) for 15 min ; the procedure was repeated. The resulting protein was washed twice with acetone, dried in a thermostat at 60°, ground in an agate mortar, and transferred in 5 mg samples to a target for counting the radioactivity.

Determination of radioactivity was carried out with a type B counter.

During the whole period of the investigation the background changed only slightly (20-23 counts per min). Determination of the radioactivity of samples and background in each case lasted 10 min; the relative error in the measurement (δ)* did not exceed 4.4%.

Desoxyribonuclease was obtained by the method of McCarty [7]. Immediately after removal, beef pancreas was put into cooled 0.25 M H_2SO_4 and put through a meat grinder, then suspended in 2 volumes of 0.25 M H_2SO_4 and extracted 12 hours in the refrigerator. The suspension was filtered through gauze and the filtrate was fractionated by saturation with ammonium sulfate. The precipitate with 0.2 saturation was rejected; the precipitate with 0.4 saturation was then fractionated between 0.17 and 0.3 saturation four times. The precipitate thus purified at 0.3 saturation contained practically all the desoxyribonuclease and was dialyzed against 0.002 M H_2SO_4 and submitted to lyophilic drying. We obtained about 500 mg of enzyme from two kg of pancreas.

We used a preparation of desoxyribonucleic acid obtained by the method of Gulland and Jordan [8].

In some of the experiments we used nuclei obtained from the thymus glands of irradiated puppies. The animals were given a total irradiation of 300 r in an RUM-3 apparatus at 180 kv, current strength 15 ma, filter 1 mm Al + + 0.5 mm Cu, strength of dose 22.5 r/ min. The puppies were killed by bleeding 24 hours after irradiation and their thymus glands were removed for separation of the nuclei. In 24 hours the animals did not lose weight; their appetite and general activity decreased somewhat.

RESULTS

At the beginning of our study we repeated the experiments of Allfrey, Mirsky, and Osawa [1] and were convinced that nuclei from calf thymus after incubation under definite conditions could take up in their protein the labeled amino acid, glycine-1-C^{14}. When DNA-ase (0.1 ml of 0.5% freshly prepared solution) was added to 200 mg of nuclei in suspension and the mixture was incubated for 45 min , and then glycine-1-C^{14} was added, the uptake of amino acid by the nuclear protein in 1 hour of further incubation was somewhat lower than in the control experiments. If, after incubation with DNA-ase, we added to the nuclei a preparation of homologous DNA (10 mg DNA in 1 ml of distilled water per 200 mg of nuclei) together with 20 mole of sodium citrate as a DNA-ase inhibitor, then the intensity of uptake of glycine-1-C^{14} by the nuclear protein in 1 hour of further incubation returned to the original value (Table 1).

We saw that the addition of DNA to the nuclei affected the uptake of glycine-1-C^{14} only when the nuclear DNA had first been damaged by DNA-ase or irradiation. Addition of DNA to the intact nuclei did not change the intensity of uptake of glycine-1-C^{14} by their proteins (Table 2).

For the following experiments, the nuclei were obtained from the thymus glands of puppies and treated with DNA-ase and just as before we found a decreased uptake of glycine-1-C^{14} in the nuclear proteins. To the incubated mixture which contained nuclei from puppy thymus treated with DNA-ase we added DNA from calf thymus and found that the uptake of glycine-1-C^{14} returned almost to the starting level (Table 3).

Thus DNA from the thymus of one kind of animal can evidently take part in restoring the cell nuclei of another kind of animal.

* The relative error in the measurement was determined by the formula : $\delta = \frac{\sqrt{\frac{n}{t} + \frac{n_\Phi}{t_\Phi}}}{n - n_\Phi}$, where n is the count of the sample in counts/ min, n_Φ the background count, t the time of measuring the sample in min, t_Φ the time of measuring the background.

TABLE 1

Effect of Adding DNA (c) on the Uptake of Glycine-1-C^{14} by Nuclear Protein (p) Submitted to the Action of DNA-ase
(intensity of uptake of glycine-1-C^{14} in counts/m in. in 5 mg protein)

Intact nuclei	Nuclei incubated with DNA-ase	% Change from control	Nuclei incubated with DNA after incubation with DNA-ase	% Change from control
241	161	−33	214	−11
211	166	−21	197	− 6
198	178	−10	196	− 1
174	149	−15	177	1.5
Average:		−19		4.1

Note: In this and later tables the letters in parentheses after "DNA" and "nuclear protein" signify: c − obtained from calf thymus, p− from puppy thymus.

TABLE 2

Effect of Adding DNA (c) on the Uptake of Glycine-1-C^{14} by Proteins of Intact Nuclei (c)
(intensity of glycine-1-C^{14} uptake in counts/ /min in 5 mg protein)

Intact nuclei (control)	Intact nuclei, incubated with DNA	% Change from control
107	103	−4
77	77	0
128	127	−0.8
78	82	5
96	93	−3
	Average:	−0.6

It was important to explain how the restoration of nuclear DNA occurred: whether there was reconstruction of the foreign DNA by first breaking it down into oligo- or mononucleotides or whether the polynucleotides of the foreign DNA entered as a whole into the composition of the nuclei which had been acted upon by DNA-ase.

If the foreign DNA was split into oligonucleotides before it entered the components of the nuclei, then it would be most likely that the nuclear DNA-ase would take part in the process. However, as we showed above, we usually added sodium citrate along with the DNA to the nuclei which had been treated with DNA-ase in order to stop the action of the latter. It would be natural to suppose that sodium citrate added in such amounts would inhibit both the DNA-ase added from outside and also that of the nuclei themselves [9]. Therefore the splitting of the foreign DNA before it was taken up by the nuclei in these experiments was unlikely.

TABLE 3

Effect of Adding DNA (c) on the Uptake of Glycine-1-C^{14} in Nuclear Proteins (p) Submitted to the Action of DNA-ase
(intensity of glycine-1-C^{14} uptake in counts/min in 5 mg protein)

Intact nuclei (control)	Nuclei incubated with DNA--ase	% Change from control	Nuclei incubated with DNA after incubation with DNA--ase	% Change from control
112	75	−33	114	1.1
118	89	−24	109	−7
170	148	−13	176	4
173	150	−14	192	10
181	150	−17	184	1.1
113	77	−32	112	−0.9
Average:		−22		0.7

In the next experiments we used x-ray irradiation as the factor which damaged the nuclear DNA. There are reports in the literature on the action of x-rays in depolymerizing DNA [11-13]. Puppies were irradiated; a day later they were killed and the nuclei were obtained from their thymus glands.

TABLE 4

Effect of Adding DNA (c) on Glycine-1-C^{14} Uptake in Nuclear Proteins (p) from the Thymus Gland of Irradiated Animals (intensity of glycine-1-C^{14} uptake in counts/m in in 5 mg protein)

Nuclei of irradiated animals (control)	Nuclei of irradiated animals incubated with DNA	% Change from controls
164	205	24
114	127	11
80	122	52
121	141	16
90	100	11
202	273	35
	Average:	24.8

The addition of DNA to the nuclei from irradiated animals considerably increased the intensity of uptake of glycine-1-C^{14} by the nuclear proteins (Table 4), while with normal animals, as we have seen, it had no effect at all on glycine-1-C^{14} uptake by the proteins (Table 2).

Thus the addition of DNA can obviously serve as an indicator of the state of DNA in the nuclei.

The literature data indicate that both DNA-ase and x-rays, acting on desoxynucleoproteins, depolymerize DNA and leave the protein intact [13, 14]. It is possible that addition of DNA to nuclei treated with DNA-ase or x-rays results in its combination with the protein of the nucleoprotein, which has partly lost its DNA, and that this restoration of the desoxyribonucleoprotein restores its chief biological properties; it is true that we do not know whether all the functions are restored, but restoration of protein synthesis certainly occurs.

A number of investigations have now established the existence of a number of differences in composition and structure of DNA [15-17]. It would be interesting to study the nature of the proteins which are synthesized by the restored nuclei which have taken up either homologous or foreign DNA. This problem remains for further investigation.

SUMMARY

It is shown that damage to DNA in nuclei taken from the thymus glands of calves or puppies by DNA-ase or by x-rays causes a decreased uptake of glycine-1-C^{14} by the nuclear proteins. This uptake is restored if the incubation medium of the nuclei is treated with DNA obtained from thymus glands of the same or different species of animals. Addition of DNA to intact nuclei does not affect the intensity of glycine-1-C^{14} uptake by nuclear proteins.

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THE CHANGE IN CERTAIN PROPERTIES OF DESOXYRIBONUCLEOPROTEINS AS A FUNCTION OF THEIR PROTEIN CONTENT

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In a previous work [1] we showed that for artifical nucleoproteins composed of serum albumin and DNA the viscosity of these complexes and their tertiary structure,* or in other words, the form of their macromolecules, remain unchanged with variations of the protein in these complexes from 350 to 750 moles/ mole DNA, that is, with a protein content in the desoxyribonucleoprotein (DNP) of about 83-93 %. As the amount of protein in the artificial nucleoproteins is increased, the relaxation properties gradually decrease, and when there are 900 moles of protein per mole of DNA (or ≃ 95% protein) they disappear entirely.

However, our artifical complexes contained a very large amount of protein while natural nucleoproteins contain less; this was because we were making a special study of the greatest possible amount of protein which could be added to the nucleic acid.

In the present study we wished to investigate changes in viscosity and relaxation in nucleoproteins which contain a relatively small amount of protein. We should expect on theoretical grounds that,as the amount of protein in DNP decreased, its viscosity and relaxation properties would change greatly, owing to the conversion of molecules of DNP to molecules of DNA [2].

METHODS

The preparation of artifical complexes of DNA-serum albumin at pH 4.5 and the measurement of viscosity and relaxation of the nucleoproteins were described by us in a previous work [1].

Natural nucleoproteins were obtained from the liver and pancreas by the method of Mirsky and Pollister [3] as modified by one of us [4, 5]; deproteinization of DNP was effected in a mixture of butanol and chloroform according to Sevag [6].

EXPERIMENTAL

Our chief experiments concerned the preparation of artificial complexes of bovine serum albumin and DNA which contained small amounts of protein, and the study of their viscosity. Under our experimental conditions, however, we did not obtain an insoluble complex with N/ P ratio less than 2.7. In this case the viscosity of the complex was about two times lower than the viscosity of the original nucleic acid, as we had found earlier [1]. Further increase in the amount of protein in the complex did not change the viscosity (Fig. 1). Thus a sharp rise in viscosity and a change in configuration of the DNA molecule when it is converted into an artificial nucleoprotein evidently occur at N/ P ratios below 2.7, that is, with less than 40% protein in the nucleoprotein molecule.

In order to study changes in viscosity in nucleoproteins with N/ P ratios below 2.7 we conducted experiments on natural nucleoproteins from beef liver and pancreas. During deproteinization of the nucleoproteins we determined the N/ P ratio, viscosity, and relaxation after each one or two treatments by the mixture of butanol and chloroform.

* By tertiary structure of nucleoproteins we mean the specific arrangement of DNP macromolecules which is formed from the tertiary structure of DNA as it combines with proteins [2].

As Fig. 1 shows, after 16 deproteinization treatments, the N/P ratio in DNP fell from 5.7 to 2.2, but the DNP viscosity remained almost unchanged.

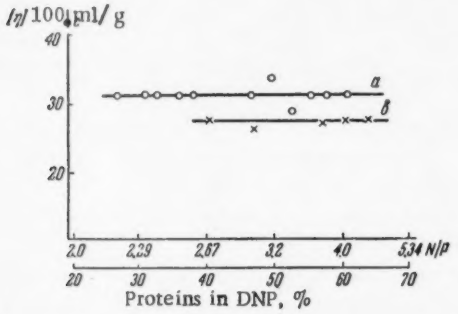

Fig. 1. Relation of viscosity characteristics to protein content in natural and artificial DNP.
a) DNP from pancreas; b) artificial DNP (liver DNA + serum albumin); c) initial viscosity of DNA which combines with protein in the artificial complex.

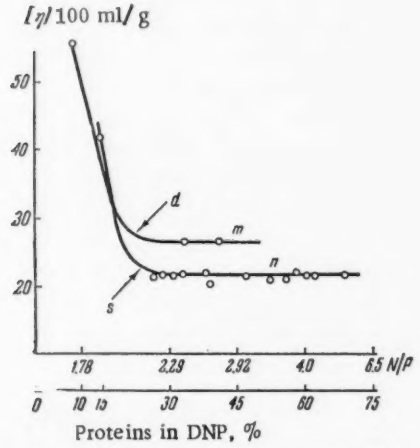

Fig. 2. Relation of viscosity characteristics of natural DNP to variations in protein content.
m) Liver DNP; n) pancreatic DNP;
d, s) points of conversion of DNP macromolecules into DNA (or DNA into DNP).

It should be noted that further deproteinization was difficult: after numerous treatments, the N/P ratio remained constant. In a number of cases after similar treatment there was a sharp fall in viscosity, probably because of depolymerization of DNP. We are forced to assume that when the N/P ratio is of the order of 2.4-2.2, only the firmly bound, or residual protein remains; this, we believe, occurs in the β-configuration and corresponds fully in its arrangment to the steric structure of DNA so that its separation becomes difficult.

Nevertheless, in spite of this difficulty, further deproteinization was possible, and then, as Fig. 2 shows, a sudden rise in viscosity was observed.

Thus the whole process of successive deproteinizations was carried out over the complete range for both pancreatic and liver DNP. The results showed that when the N/P ratio changed from 2.2-2.4 to 1.80-1.90 the protein content fell from 27-34% to 11-17% and the viscosity of the samples almost doubled.

It should be noted that after deproteinization the molecular weight of the resulting DNA was different, but independent of this factor, the qualitative and quantitative effects on change of viscosity were always about the same.

The results of a study of the relaxation properties of liver DNP during deproteinization are given in Fig. 3. We see that with decrease in N/P ratio from 5.7 to 4.1 the relaxation coefficient falls slightly, and then below a ratio of 3.9-4.1 there is a rise, and the relaxation properties suddenly disappear; this always occurs at the same N/P ratio in spite of the wide range of DNP concentrations in the samples and independent of whether the DNP was obtained from liver or pancreas [5].

Similar disappearance of relaxation with decreased amount of protein was found with the artificial complex from DNA and bovine serum albumin (Fig. 3). In this case the loss in ability to relax occurred at N/P ratio 3.4-3.8, about the same ratio as for natural DNP. However, in general for the artificial complex to relax, its concentration in the sample should be somewhat higher than in natural DNP.

When we compare these data on relaxation of DNP with low protein content and our earlier results [1] on relaxation of artificial DNP with high protein content, we obtain a theoretical curve of relaxation for artificial complexes with all ratios of their protein (Fig. 4).

DISCUSSION

Our failure to obtain insoluble artificial complexes with N/P ratios below 2.7 is probably due to the fact that the insolubility of the complexes depends on aggregation of the molecules of DNP and the formation of a macro-

molecular structure. However, this formation is possible only when there is a sufficient concentration of DNP in solution and a definite minimum protein content in the nucleoprotein; below this, evidently, at least in any practical concentration, there is no reaction between molecules of DNP. When the protein content of the complex is on the order of 15-25%, the complex will occur in the fully stretched β-configuration and the acid groups of the polypeptide chain extend outward, that is, there will be repulsion between the similarly charged surfaces of the DNP molecules. At higher concentrations of protein in the complex the arrangement should be less rigid, and since all the phosphorus groups of DNA in this case would be combined, some of the basic groups of the protein would remain free. As a result, the charges on different parts of the surface of the DNP molecule would differ and this would favor aggregation. On the other hand, with increase in protein in DNP, the latter will be dehydrated, and this also permits its precipitation.

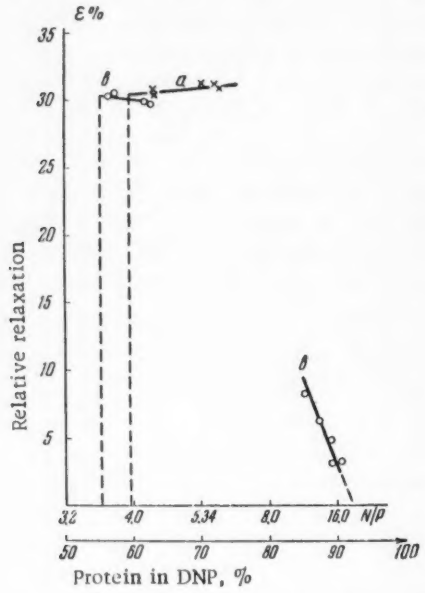

Fig. 3. Relation of relative relaxation of natural and artificial DNP to protein content.
a) Pancreatic DNP; b) artificial DNP (liver DNA + serum albumin).

The constant value of the viscosity of DNP when the protein content varies widely shows the great stability of the configuration of the DNP macromolecule. It must be assumed that a considerable part of the protein in the nucleoprotein is bound to it in such a way that it does not determine the configuration of its tertiary structure and can be removed without affecting the DNP molecule.

However, continued removal of protein from DNP leads to inversion of its macromolecular structure and the form of its molecule approaches the form of the DNA molecule. We think that there is a qualitative change, and the molecule of DNP is changed into a DNA molecule. We have already indicated the existence of such a change in our previous work [2]. Probably, protein in the amount of about 15-20% in DNP blocks positions of rupture in the DNA molecule, and it is held in steric positions which produce a qualitatively new structure, inherent in the DNP already.

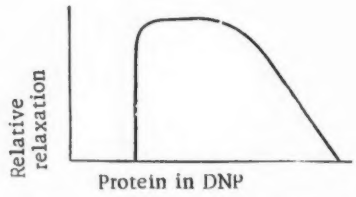

Fig. 4. Generalized (theoretical) relation of relative deformation of nucleoprotein to its protein content.

It is true that the sudden rise in viscosity when full deproteinization of nucleoprotein occurs and when artificial complexes are obtained had been noticed by other authors, as we saw from our review [2], but the actual amount of protein at which this rise occurs had not been quantitatively determined. At the same time, Henstell and co-workers [7] showed, on the basis of indirect evidence, namely, the changes in denaturation of DNP with different protein content, that about 18% protein determines the properties and structure of DNP.

Measurements of relaxation reflect the effects of macromolecular structure in DNP and the formation of intermolecular bonds. Experimentation shows that to produce these effects there must be a large enough amount of protein in the DNP, and the effect of concentration at the minimum protein does not have much significance.

From a consideration of the theoretical curve which relates relaxation to the amount of protein in DNP (Fig. 4) we can conclude that the general form of the curve remains unchanged, but it can be shifted to one side or the other depending on the concentration of DNP in the sample and perhaps on the manner in which the protein is combined in the nucleoprotein.

At first the amount of protein in the DNP is clearly not sufficient to permit formation of intermolecular bonds, and relaxation of DNP, as well as structure, is absent; then, as the amount of protein increases the molecules

begin to react with each other forming a quaternary* macromolecular structure which is capable of relaxation. As the amount of protein in the DNP increases further, there is a "cementation" effect at points where the spiral of DNA would rupture, so that it becomes more stable, and also the number of intermolecular bonds increases so that the structure becomes stronger. Relaxation begins to decrease, at first slowly, then faster, and finally, when all the bonds around which internal rotation can occur become fixed, and, of course, the cross linkages increase sharply, it ceases entirely. Thus, at very small or very large amounts of protein in DNP, relaxation is absent, but for different reasons in each case.

In this way we have shown that the specific properties of nucleoproteins – their lower viscosity than that of DNA and their ability to undergo relaxation (DNA scarcely relaxes) – depend on the amount of protein in the DNP, so that as any given property occurs, there is a corresponding relation to the protein content of the DNA.

We have found three inverstion points. One, at a protein content of 15-25%, corresponds to the appearance of the specific tertiary structure in the macromolecules of DNP; another, at 58-62% protein (N/P $\simeq$ 3.8-4.1), corresponds to the appearance of structural and mechanical properties which occur when the quaternary macromolecular structure of DNP is formed. It is interesting that a number of authors, as we mentioned in our review [2] think that after DNP from different sources has been purified it has the characteristic N/P ratio $\simeq$ 3.8-4.1. Finally, the third inversion point is characterized by complete loss of the highly elastic properties of DNP at a protein content of 90-95%.

We consider that the effects of the tertiary macromolecular structure of DNP are the most important and the most decisive for the nucleoprotein molecule. Therefore, we must consider as nucleoproteins those compounds of protein and DNA which contain 15-25% protein.

SUMMARY

We have studied changes in viscosity and relaxation of DNP depending on the amount of protein which it contains.

We have found by direct experiment the amount of protein which corresponds to change of the macromolecule of DNA to the macromolecule of DNP. It is about 15-25%.

We have shown that in order to produce certain structural mechanical properties of DNP (in concentrations of 0.01-0.2%) the protein content must be 58-62%.

When the DNP contains 90-95% protein these properties once again disappear (as in the case of pure DNP), although the configuration of the molecule does not differ from that of DNP with a smaller protein content.

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* By quaternary structure of DNP we mean a macromolecular structure which results from formation of bonds between molecules of DNP and which gives a whole new set of structural mechanical properties [2].

** Original Russian pagination. See C. B. Translation.

THE EFFECT OF ORGANOPHOSPHORUS INSECTICIDES ON ASCORBINOXIDASE

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We have investigated the effect of a number of organophosphorus compounds on the oxidase of ascorbic acid of apple leaves in vitro.

The enzyme activity was determined by the method of Bukin [1] with a variation in the composition of the components and their proportion in the titrated mixture. The mixture titrated with 2,6-dichlorophenolindophenol contained 2 ml of buffer (pH 5.5), 0.5 ml of insecticide solution or emulsion, 2 ml of enzyme suspension, 0.5 ml of ascorbic acid solution (0.2 mg), and 0.5 ml of 2% hydrochloric acid solution. The dilution of the original portion was 1 : 120. The incubation period was 15 minutes.

For the preparation of the emulsions or solutions of the insecticides, starting preparations were used that contained (in addition to the toxic substance) alkylphenyl ethers of polyethylene glycol (accessory agent OP-7). Most of the starting preparations contained 70% OP-7; the technical compound Methylsystox contained 40%, and the compound M-81 contained 50% OP-7.

In the table are given the compounds tested and their molar concentrations at which 50% inhibition of the ascorbinoxidase activity occurred (I_{50}). The values of I_{50} were calculated graphically from the titration results.

Concentrations of Organophosphorus Insecticides Causing 50% Inhibition of Ascorbinoxidase Activity

Compound	I_{50} in $M \cdot 10^3$ *
O,O-Diethyl S-ethyl β-mercaptoethylthiophosphate – thiol isomer of Systox (pure)	1.3
O,O-Diethyl O-ethyl β-mercaptoethylthiophosphate – thiono isomer of Systox (pure)	1.3
O,O-Dimethyl S-ethyl β-mercaptoethyldithiophosphate – compound M-81 (technical)	1.0
Mixture of thiono and thiol isomers of Systox (3 : 1) – Mercaptophos (technical)	1.3
O,O-Dimethyl O-ethyl β-mercaptoethylthiophosphate – Methylsystox (pure)	1.5
Same (technical)	2.0
O,O-Diethyl O-p-nitrophenyl thiophosphate – NIUIF-100 (pure)	0.6
Same (technical)	0.7
O,O-Methylethyl O-p-nitrophenyl thiophosphate – Methylethylthiophos (pure)	0.7
Same (technical)	0.9
O,O-Dimethyl β,β,β-trichloro-α-hydroxyethylphosphonate – Chlorophos (pure)	3.8

All of the organophosphorus insecticides investigated proved to be closely similar in their inhibiting effect on the ascorbinoxidase, although O,O-dimethyl β,β,β-trichloro-α-hydroxyethylphosphonate (Chlorophos) was somewhat less toxic.

No increase was observed in the ascorbinoxidase activity under the influence of the compounds tested (at concentrations $1 \cdot 10^{-4}$ and $1 \cdot 10^{-2}$ M).

Determination of the ascorbinoxidase activity by the method employed did not reveal substantial differences between the various compounds taken in equal concentrations. The amount of 2,6-dichlorophenolindophenol con-

* As in original.

sumed in the titration of the ascorbic acid that remained unoxidized varied for a single determination within the limits ± 0.12 ml. The accessory material OP-7 also was capable of depressing the activity of the ascorbinoxidase; OP-7 gave I_{50} in a concentration ~0.01%.

The sensitivity of the ascorbinoxidase in relation to the organophosphorus insecticides tested was less than that of cholinesterase (I_{50} corresponds to $1 \cdot 10^{-4} - 1 \cdot 10^{-8}$ M [2; 3]); the inhibition of the activity of cytochromoxidase by O,O-diethyl O-p-nitrophenyl thiophosphate and O,O-dimethyl α,β-dicarboethoxymethyldithiophosphate is of approximately the same order ($1 \cdot 10^{-3}$ M [4]) as ascorbinoxidase.

SUMMARY

The organophosphorus insecticides O,O-diethyl S-ethyl β-mercaptoethylthiophosphate (I), O,O-diethyl O--ethyl β-mercaptoethylthiophosphate (II), O,O-dimethyl S-ethyl β-mercaptoethyldithiophosphate, a mixture of I and II in a ratio of 1 : 3, O,O-dimethyl O-ethyl β-mercaptoethylthiophosphate, O,O-diethyl O-p-nitrophenyl thiophosphate, and O,O-methylethyl O-p-nitrophenyl thiophosphate proved to be very similar in their inhibitory action upon ascorbinoxidase of apple leaves in vitro. O,O-dimethyl β,β,β-trichloro-α-hydroxyethylphosphonate was somewhat less toxic.

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THE USE OF A DENSITOMETER FOR QUANTITATIVE DETERMINATION OF CATECHINS SEPARATED BY PAPER CHROMATOGRAPHY

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The present work was undertaken to provide an easy and rapid method for the quantitative determination of catechins. The catechins were separated by descending paper chromatography.

A new recording densitometer was used for the quantitative estimation; it was developed by one of us expressly for the quantitative determination of various substances which had been separated by paper electrophoresis.

Tannins are among the most important substances in the tea plant, and so they have been studied by many investigators [1-4]. However, these studies have been based mostly on determination of total tanning substances by the permanganate method [5] or by the determination of separate fractions which were actually mixtures of catechins.

Bradfield [6] and Zaprometov [7] separated a number of catechins from the tanning substances of tea by the column chromatography method. These studies showed the importance of a more careful study of a number of problems of the biochemistry of the tanning substances in tea plants, such as the formation and transformation of catechins, their changes in the developing leaf during growth, the variations in catechins during production of different types of tea, and so on. These problems require more than anything else a reliable and simple method for the determination of catechins. Dzhemukhadze and Shal'neva [8] worked out a quantitative method for catechins by paper chromatography followed by elution and colorimetric determination in the resulting solutions. This opened the way to a number of new studies. However, the method was relatively difficult and lengthy.

For the chromatographic separation of the catechins we used paper which was cut as directed by Mathias [9] into strips 250 × 30 mm with a neck 10 × 3 mm. For the solvent we used a mixture of n-butanol–glacial acetic acid–water in the ratio 40 : 12.5 : 32. After the separation the chromatogram was dried for 1 hour under a hood at room temperature and then developed with a 1% ammoniacal $AgNO_3$ solution.

Dzhemukhadze and Shal'neva developed their control chromatogram with a 1% solution of vanillin in concentrated HCl. This developer was not suitable for our method because it decolorized rapidly on the chromatogram. Therefore we studied a number of compounds which gave colors with catechins, and we finally chose 1% ammoniacal silver nitrate. The spots on the chromatogram which contained catechins gave dark brown color with this solution. Systematic measurement of the intensity of the color showed that when the chromatogram was kept in the dark, there was no evident change during 24 hours.

The sensitivity of the reaction was 0.3 μg of catechin. A difficulty was that under the action of light the silver was reduced on the paper and the chromatogram darkened in light. Attempts to prevent this by treating the developed chromatogram with 25% sodium thiosulfate gave negative results, since besides fixation of the white background of the chromatogram there was a considerable change in the intensity of spot color. Therefore all further drying of the developed chromatogram and its preservation took place in the dark.

Quantitative determination of the chromatographically separated catechins was carried out on a photoelectric densitometer. The chief features of this apparatus and its general appearance are given in Figs. 1 and 2.

The apparatus permits an objective quantitative determination of the distribution of substances by direct

measurement of the intensity of color in the spots on the paper. The measurements are made with transmitted light and the results obtained as a curve which indicates the amount of light absorption for each spot which is measured.

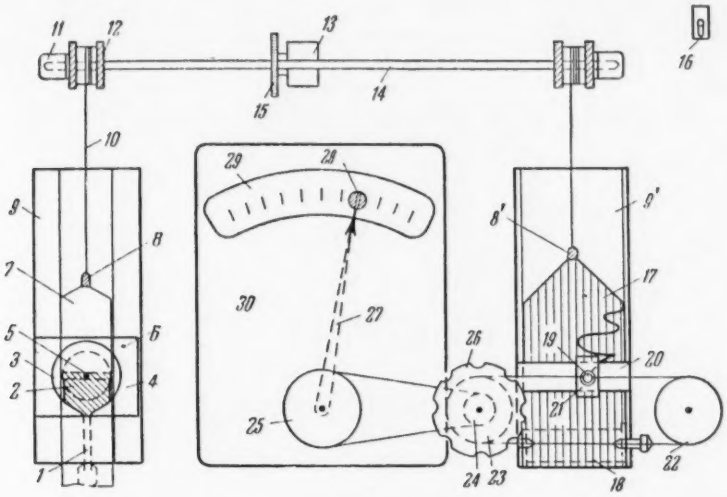

Fig. 1. Plan of densitometer.

1) Diaphragm; 2) light source (incandescent lamp); 3) photoelement; 4) ring for photoelement; 5) slot; 6) photoelement switch; 7) paper strip (chromatogram); 8, 8') clamps; 9) casing for measuring chamber; 10) wire to winding mechanism; 11) coupling shaft; 12) spool; 13) motor driving gear; 14) axis; 15) reducing gear; 16) switch; 17) diagram paper; 18) spool for diagram paper; 19) recording pen; 20) guide adjustment; 21) guide; 22, 23, 24, 25) sheaves; 26) shaft for "following" mechanism; 27) pointer for "following" apparatus; 28) luminous indicator of galvanometer; 29) glavanometer scale; 30) galvanometer.

The strip of paper 7 with the spots on it is put between the light source 2 and the photoelement 3. The deflection of the galvanometer indicator is established by the diaphragm 1 on all the scales of the apparatus, that is, 100% passage of light is established for a "clear" portion of the paper strip (free from substances). When the winding mechanism is turned on, the paper strip is drawn at constant rate past the slot 5 off the densitometer. The diagram paper 17 is synchronized with it. When the spots caused by any substance are passed through the slot, light is absorbed to produce a change in the photocurrent and a corresponding deflection in the galvanometer indicator. The shaft 26 transmits the action of the apparatus 27 (which repeats the movements of the galvanometer indicator 28) and the pen 19 in the recording part of the apparatus, which is connected mechanically with the arrow of the following mechanism, traces the corresponding curve on the diagram paper. This method gives a semiautomatic record of the movements of the galvanometer 30. If use is made of a recording galvanometer with a sensitivity of $1 \cdot 10^{-8}$ to $5 \cdot 10^{-9}$ amp. or an electronic self-registering potentiometer of type EPP-09, recording of the curve is completely automatic. The densitometer is provided with a set of light filters which cover the visible spectrum so that measurements can be made in more or less monochromatic light.

Our chromatograms, treated with 1% ammoniacal $AgNO_3$, were measured on the densitometer with a blue light filter which had maximum light transmission at 430 mμ.

Quantitative calculations were carried out by usual planimetric methods from the form of the absorption curve of the measured substance and the line of 100% transmission of light by the pure paper.

Numerous tests showed that the experimental error of the apparatus itself, caused by so-called "cloudiness of the paper," stability of the light current, fatigue of the photoelement, and other factors, did not exceed ± 5%. The appearance of the chromatogram and the absorption curves of the catechins on the paper, as determined with the densitometer, are shown in Fig. 3.

A calibration curve was necessary for the quantitative determination of catechins by the densitometer. For this purpose known amounts of catechins were placed on the chromatogram and after development they were measured on the densitometer. In the calibration curve the ordinate represented the area of absorption curve in mm^2 and the abscissa the concentration of catechin in micrograms.

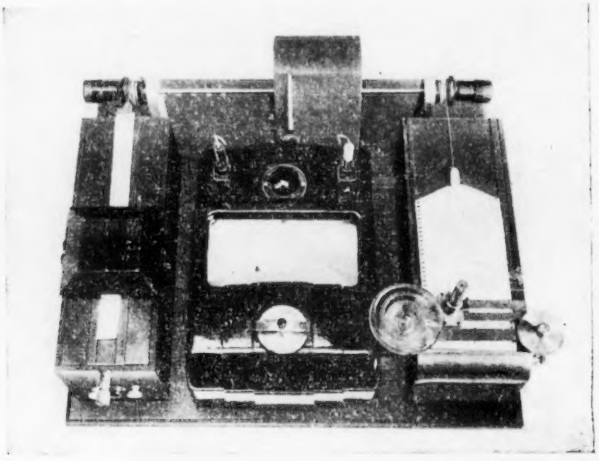

Fig. 2. General appearance of densitometer.

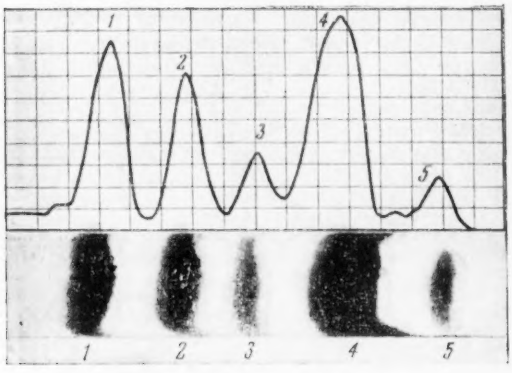

Fig. 3. Appearance of chromatogram and absorption curve for various substances:
1) L-epigallocatechin; 2) DL-gallocatechin; 3) DL-catechin + epicatechin; 4) L-epigallocatechingallate; 5) L-epicatechingallate.

Content of Catechins in Tea Sprouts

Catechin in mg/g dry weight	Sprout	First leaf	Second leaf
L-epigallocatechin	9.3	25.2	42.5
DL-gallocatechin	trace	4.2	4.5
DL-catechin + L-epicatechin	trace	3.5	5.1
L-epigallocatechingallate	47.5	41.6	53.5
L-epicatechingallate	26.7	30.0	30.2

An example of the calibration curve for L-epigallocatechingallate is given in Fig. 4 (the preparation was kindly supplied to us by M. P. Zaprometov).

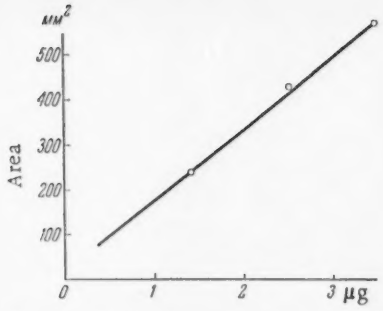

Fig. 4. Calibration curve for L-epigallocatechingallate .

Since we had no chemically pure catechins, we were forced to construct our calibration curves for the catechins with standard solutions of tea tannins in which the content of the different catachins had been determined by the method of Dzhemukhadze and Shal'neva. Definite volumes of solutions with known amounts of the catechins were then placed on the paper. The concentration placed on the paper and the corresponding area of the absorption curve were used to construct the calibration curve.

This method was used to determine the catechins in tea sprouts.

For this purpose 20 ml of hot distilled water was poured over 0.5 g of dry material and extraction was continued on the boiling water bath for 20 min. Then the extract was diluted to 25 ml and the solution was filtered. For each determination we used 4 ml of extract. Further determination of the catechins was carried out by the method described above.

The results given in the table show that in all parts of the tea sprout L-epigallocatechingallate predominates. A consideration of the changes in content of L-epigallocatechin, DL-catechin, L-epicatechin and DL-gallocatechin shows that the amounts of these catechins increase with the age of the tea leaves. The results fully agree with those of Dzhemukhadze and Shal'neva [10] which were obtained by another method.

SUMMARY

A method has been developed for the quantitative determination of catechins by separation on paper chromatograms and subsequent estimation with the densitometer of M. S. Shipalov.

The catechins in the parts of tea sprouts have been determined by this method.

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THE ROLE OF SUCROSE IN THE LEAVES OF "GLUCOSIDE" PLANTS

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When sucrose is formed in plants, two cyclic forms of monosaccharides are fixed – α-glucopyranose and β-fructofuranose; thus the disaccharide may take part in transglucosidal or transfructosidal reactions [1, 2], and it may react not only as a donor, but as an acceptor of fructose [3]. Thus sucrose takes part in the activation and control of the reactions of the most widely distributed monosaccharides, glucose and fructose [3, 4]. Sucrose also must play an important part in their decomposition, since it is a source of the unstable furanose form of fructose [5, 6].

In the leaves of the lilac (Syringa vulgaris) and the ash (Fraxinus excelsior) the "sucrose fraction," according to our data, has a complex composition and consists of sucrose, fructosides, and easily hydrolyzed glucosides (L--glucosides) [7]. The most dynamic components of this fraction are the L-glucosides. The sucrose content in the leaves of these plants, as opposed to that in the leaves of the majority of plants, is almost unchanged during a day of photosynthesis or with long keeping in the dark, though the invertase activity is unusually high [8]. Infiltration of monosaccharides into the leaves of these plants never causes much increase in the amount of sucrose, although the infiltrated monosaccharides are used up very quickly [9].

Such unusual behavior of sucrose made it interesting to conduct experiments on its infiltration into the leaves of the "glucoside" plants to discover how rapidly it was used up and its effect on the content of the other components of the "sucrose fraction."

EXPERIMENTAL

Sucrose solution was infiltrated into the leaves of lilacs and ash by the method of Kursanov [7]. After infiltration the leaves were fixed with steam and analyzed by the method which was previously described and which permitted determination of all three components of the "sucrose fraction" [7]. The sum of the water-soluble carbohydrates which were relatively hard to hydrolyze (glucose S-glucosides) was determined by the increase in reducing sugars after 3 hour hydrolysis by 2% HCl on the boiling water bath.

Experiment I. A 0.1 M solution of sucrose was infiltrated into 12 g of fresh lilac leaves. After removal of intercellular water the leaves were put into a moist desiccator for 2 hours, then fixed with steam and analyzed. In one series of experiments the leaves were analyzed immediately after infiltration and before removal of water from the intercellular spaces.

The results are given in Table 1.

The data which were obtained show that the sucrose infiltrated into the leaves was used up extremely rapidly. Even immediately after infiltration the increase in sum of monosaccharides and sucrose together was 3.2% though the weight of leaves after removal of water from the intercellular spaces showed an increase of 4%. After 2 hour exposure, about half the added sucrose remained in the leaves in the first experimental series only; in series 2 about 4/5 of the infiltrated sucrose was used, and in series 3 almost all the sucrose was used.

The results indicate that sucrose takes an active part in the carbohydrate metabolism of "glucoside plants" as one of the most important intermediate compounds. The relatively low content of sucrose, which is almost unchanged in different processes in the leaves, is explained by the fact that the synthesis and breakdown of this

disaccharide go at the same rate. The small change in monosaccharide content when sucrose is infiltrated shows that the chief path for sucrose is not through the formation of monosaccharides by hydrolysis, but through the rapid transfructosidal reaction which is accompanied by liberation of glucose. It must be remembered that monosaccharides are very rapidly used up in the leaves of "glucoside plants" [9]. Perhaps the monosaccharides formed when sucrose is split are used very rapidly and so their content does not change. The content of fructosides and difficultly hydrolyzable compounds also changes little.

TABLE 1

Infiltration of 0.1 M Sucrose Solution in Lilac Leaves

Values expressed in % dry weight

Carbohydrate fraction	Experimental data						
	7/6 (1)			9/6 (2)		9/28 (3)	
	Before infiltration	Directly after infiltration	2 hours after infiltration	Before infiltration	2 hours after infiltration	Before infiltration	2 hours after infiltration
Fructose	1.7	1.5	2.3	2.1	2.2	2.0	2.2
Glucose	0.5	1.2	0.9	0.2	0.0	0.4	0.8
Sum of monoses	2.2	2.7	3.2	2.3	2.4	2.4	3.0
"Sucrose fraction"	5.5	7.7	6.3	4.5	4.4	4.4	3.9
Sucrose	1.3	4.0	3.3	1.2	2.0	1.3	1.4
Glucose L-glucosides	4.2	3.5	2.4	2.8	1.9	2.4	2.2
Fructosides	0.0	0.3	0.6	0.5	0.5	0.6	0.3
Glucose S-glucosides	7.4	6.5	8.0	8.8	9.0	6.3	6.1
Sum of water soluble carbohydrates	15.1	16.5	17.5	15.6	15.7	13.1	13.0

The amount of labile glucosides falls in all the experiments, sometimes greatly. Evidently the components of the "sucrose fraction" have a definite effect on each other and on the equilibrium which depends on the relative rate of the rapid processes of formation and further reaction of these labile compounds. It may be that in the presence of excess sucrose, which contains α-glucose, the rate of synthesis of the labile glucosides, which contain β-glucose, is somewhat decreased.

TABLE 2

Infiltration of 0.1 M Sucrose Solution into Lilac Leaves after Preliminary Keeping in the Dark

Values expressed in % dry weight

Carbohydrate fraction	Before infiltration	2 hours after infiltration
Fructose	2.0	2.4
Glucose	0.4	0.1
Sum of monosaccharides	2.4	2.5
"Sucrose fraction"	2.9	4,2
Sucrose	0.8	1.2
Glucose L-glucosides	1.8	2.4
Fructosides	0.3	0.6
Glucose S--glucosides	8.0	7.3
Sum of water soluble carbohydrates	13.7	14.6

The sum of the water-soluble carbohydrates is somewhat increased in one series, and is almost unchanged in the others. This indicates that sucrose also takes part in the synthesis of substances which are not sugars.

The next experiment was carried out with lilac leaves which were first kept in the dark.

Experiment II. Lilac leaves collected September 7 were kept in the dark in amoist desiccator for 48 hours before infiltration, a procedure which decreased the content of the "sucrose fraction" considerably, mostly in the labile glucosides.

The results are given in Table 2.

The results of Table 2 confirm the data of Experiment I, showing the rapid use of sucrose for synthesis of substances which are not sugars. The content of monosaccharides and other specific carbohydrates changes only slightly. The labile glucosides are an exception; their content, artificially decreased by a long period

in the dark, is somewhat increased. This shows that some monosaccharides are formed in the reactions of sucrose, since otherwise it would be difficult to account for conversion of α-glucose into β-glucose. The monosaccharides, whose solutions contain all the isomeric forms, evidently occupy a central position in carbohydrate metabolism. The reactions of different forms of hexoses begin with the formation of one or another monosaccharide, fixed in one of its isomeric forms.

Since we found in most of the experiments a very rapid use of sucrose without accumulation of monosaccharides, we thought it would be interesting to carry out experiments with infiltration of more concentrated sucrose solutions and shorter periods of keeping after infiltration.

Experiment III. Lilac leaves collected at 10 A.M. were infiltrated with 0.14 M sucrose solution and kept 1 hour in the desiccator.

Results are given in Table 3.

TABLE 3

Infiltration of 0.14 M Sucrose Solution into Lilac Leaves

Values expressed in % dry weight

Carbohydrate fraction	Experimental data				
	6/16			10/7	
	Before infiltration	1 hours after infiltration	Before infiltration	1 hour after infiltration	
				1	2
Fructose	1.4	1.1	2.0	2.6	2.3
Glucose	0.4	1.5	0.6	0.6	0.4
Sum of monosaccharides	1.8	2.6	2.6	3.2	2.7
"Sucrose fraction"	7.9	8.1	3.7	6.1	5.3
Sucrose	1.0	1.8	1.2	3.4	3.1
Glucose L--glucosides	5.4	4.6	2.5	2.5	2.2
Fructosides	1.5	1.7	0.0	0.2	0.0
Glucose S--glucosides	7.3	5.7	10.9	10.0	10.6
Sum of water soluble carbohydrates	17.0	16.4	17.2	19.3	18.6

The data of Table 3 once more confirm the results of the previous experiments which showed the extremely rapid use of sucrose infiltrated into the leaves of "glucoside" plants. This process went especially fast in the leaves collected in June. Although in the experiments with infiltration of 0.14 M sucrose solution we introduced up to 50 mg sucrose per 1 g of dry leaf material, the increase in sucrose was only about 0.8%. Evidently most of the sucrose was used here in the synthesis of substances which were not sugars, since the content of labile and difficultly hydrolyzed glucosides did not increase, but even fell slightly. Sucrose was used up somewhat more slowly in the autumn, but here also about half the infiltrated sugar was used to synthesize substances which were not sugars or to undergo decomposition.

The next experiments were made with ash leaves, which contained more sucrose and less labile glucosides than did the lilac leaves.

Experiment IV. The ash leaves, collected at 10 A.M., were infiltrated with 0.1 M sucrose solution and the leaves were kept in the desiccator for 2 hours.

The results are given in Table 4.

The results from infiltration of ash leaves by sucrose differ considerably from those obtained with lilac leaves. Although infiltration did not succeed as well as in the experiments with lilac, the increase in sum of water-soluble carbohydrates was more than 4%; this corresponded to the whole amount of sucrose introduced into the leaves. The increase of monosaccharides in this experiment was slight, and most of the infiltrated sucrose was used in the synthesis of relatively difficultly hydrolyzable glucosides. There was also some increase in labile glucosides.

The synthesis of glucosides from most of the sucrose evidently precedes the formation of monosaccharides, since otherwise it would be difficult to explain the change of α-glucosides into β-glucosides.

TABLE 4

Infiltration of Sucrose Solution into Ash Leaves

Values expressed in % dry weight

Carbohydrate fraction	Experimental data			
	7/7		9/13	
	Before infiltration	2 hours after infiltration	Before infiltration	2 hours after infiltration
Fructose	1.1	1.2	1.3	1.8
Glucose	1.0	0.9	0.5	0.3
Sum of monosaccharides	2.1	2.1	1.8	2.1
"Sucrose fraction"	6.2	8.3	5.7	7.1
Sucrose	4.1	5.4	3.1	4.5
Glucose L--glucosides	1.7	2.4	1.9	2.0
Fructosides	0.4	0.5	0.7	0.6
Glucose S--glucosides	5.6	8.3	8.4	10.9
Sum of water soluble carbohydrates	13.9	18.7	15.9	20.1

The experiments with lilac and ash leaves thus showed extremely rapid use of sucrose accompanied by no accumulation of monosaccharides; this confirms the earlier idea [8, 9] of the important and partly independent role of sucrose and the labile glucosides in carbohydrate metabolism as intermediate reactive compounds which are easily formed and which easily undergo further reaction. When these compounds are formed, there occurs fixation of a specific form of monosaccharide which determines the direction in which the compound is used for various later purposes. It is quite possible that sucrose is the precursor and that its synthesis occurs from phosphates or other compounds of the hexoses (such as uridine phosphate glucose [11-14]).

Kursanov and co-workers showed that sucrose and its precursors play an important part in the development of sugar beets [11, 15] and also in cotton when the plastic substances in the fibers are used for the synthesis of cellulose [13]. It seems to us that in the latter case there is a preliminary splitting of sucrose to monosaccharides or their derivatives with free glucoside carbons. The results of Pavlinova favor this view, for they show that synthesis of sucrose does not occur in the roots of sugar beets, but when monosaccharides are introduced into the roots they undergo rapid transformation and, in the opinion of this author, they are used to form cellulose and pectins, and for respiration [16].

It is probable that simple hydrolysis of sucrose into monosaccharides does not happen very often, since this reaction is not favored from the energetic point of view; instead occur the reactions of the transglucosidal or transfructosidal type in which the energy of one glycoside bond is used and a monosaccharide is liberated from the sucrose [17-19]. The formation of a certain amount of monosaccharide plays an important part in carbohydrate metabolism, since only in this way is it possible to go from a compound containing α-glucose to β-glucose, or the reverse. This has great significance since recent data indicate the synthesis of β-oligosaccharides by a transglucosidal reaction [20, 21].

Hexoses whose solutions are in dynamic equilibrium between the different forms probably occupy a central position in carbohydrate metabolism. They form the central reserve of all the labile isomers whose reactivity makes possible their increased use; their dynamic state permits different forms of the hexose to react at the moment of formation or even during the time of transition from one form to another. Such an equilibrium is also possible between the monosaccharide phosphates if the glucoside carbon is free [22].

The wide participation of monosaccharides in carbohydrate metabolism is also shown by their good assimilation by different organisms, and also by the discovery of mutarotation in preparations of glucose oxidase from Penicillium notatum [23].

It is interesting that only the hexoses give the quilibrium between all these forms. In the pentoses the length of the carbon chain is not sufficient for the formation of the pyranose form of the ketopentoses, and heptoses have no advantage over hexoses in this respect.

At the present time there is evidence that one of the first products of photosynthesis is heptulose which is transformed into a pentose. The trioses and sucrose are formed [24].

Many authors consider that sucrose is the first free sugar formed in photosynthesis [25]. Nevertheless, glucose and fructose are the most widely distributed sugars and those most easily assimilated by all living organisms. It is possible that this is related to the dynamic equilibrium which has been discussed.

SUMMARY

Sucrose infiltrated into lilac and ash leaves is used up very rapidly but an appreciable increase in monosaccharides does not occur.

The utilization of sucrose in lilac and ash leaves differs. In the first, most of the infiltrated sucrose is used to form substances which are not sugars, in the second it is used for the synthesis of definite carbohydrates, chiefly the "difficultly hydrolyzable glucosides." Their synthesis is evidently preceded by formation of free glucose, whose β-form combined with an aglycone.

In the experiments with lilac the infiltration of sucrose often decreases the amount of labile glucosides, apparently by slowing their synthesis.

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THE SOURCES OF METHYL GROUPS IN THE FORMATION OF N'-METHYLNICOTINAMIDE IN THE ORGANISM

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The chief end product in the metabolism of nicotinic acid (NA) in carnivorous and omnivorous animals is N'-methylnicotinamide (MNA); in humans and some mammals a definite part of it also appears as N'-methyl--6-pyridone-3-carboxamide.

The question of the origin of the methyl groups during the biological methylation of NA has not been completely solved.

When liver sections were incubated with nicotinamide, they could synthesize MNA; addition of methionine intensified this synthesis in vitro (though not in all cases) [1]. An enzyme was isolated from liver, nicotinamide methylkinase which catalyzed the formation of MNA from nicotinamide in the presence of L-methionine, magnesium ions, and adenosine triphosphate [2]. It was also shown that when NA was methylated in vivo, methionine served as a source of methyl groups, but betaine did not [3, 4]. While one author considered that choline contributed its methyl group directly in the biosynthesis of MNA [5] another denied this possibility and believed that its CH_3 groups could participate only when homocystine was added simultaneously. It was also shown that vitamin B_{12} could intensify the biosynthesis of MNA [4].

The question arises whether only a source of active methyl groups (methionine) which comes from the diet can be used for the synthesis of MNA. There is no doubt that the intestinal flora take part in the formation of active methyl groups, but this path does not have much significance; when the activity of the intestinal flora is inhibited by sulfonamides, the excretion of MNA in rat urine is only decreased, but is not stopped altogether [6].

To settle the question of whether the MNA synthesis is due to endogenous sources of the methyl group we carried out two series of experiments. The question was settled positively.

EXPERIMENTAL

In the first series, protein was entirely eliminated from the diet of rats, and so not only methionine was missing, but also the amino acids (glycine, serine) from which ethanolamine is formed. In the second series the animals were starved completely.

The experiments were carried out on male white rats which weighed 150-170 g. The diet which contained protein (I) had the following composition: 18% casein, 63% corn starch, 14% sunflower oil, 5% salt mixture. Dry brewers yeast, 0.5 g a day per rat, served as a source of B vitamins. The animals received 20 I.U. of vitamin A and 8 I.U. of vitamin D per day, and also 1 mg of vitamin E daily.

The diet which lacked protein (II) contained instead of casein a calorifically equivalent amount of starch. The animals were kept in individual cages with glass lattice bottoms; the urine was collected separately from the feces through a special funnel. The amount of MNA in the urine was determined by the method of Huff and Perlzweig [7] after a dose of nicotinic acid amide (NAA) or nicotinic acid. The solution of NAA or NA was placed in the throat by a probe (20 mg in 0.5 ml of distilled water).

Series I. Protein Deficiency

In the preliminary period, five rats were given diet I which contained protein and at the same time 20 mg of NAA; the amount of MNA excreted in the urine was determined daily. When the increase in MNA due to the added NAA had returned to the starting level, which was determined before the addition, protein was removed from the diet of the rats. At the end of 32-38 days the protein-starved rats (diet II) were again given 20 mg of NAA and the protein-free diet was continued for three more doses at intervals of 6-8 days, at first NA, then NAA, then NA again.

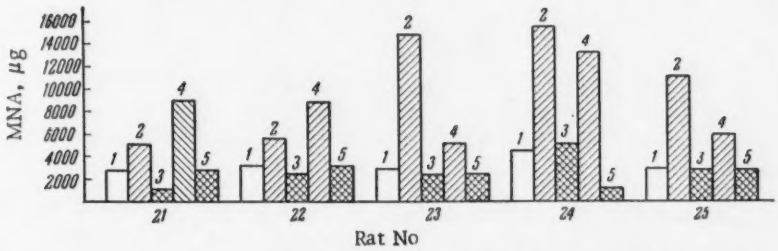

Fig. 1. Excretion of N'-methylnicotinamide in the urine for 3 days after doses of NAA or NA:
1) Dose of NAA in a diet which contains protein; doses in protein-free diets:
2) NAA (17-38 days); 3) NA (23-43 days); 4) NAA (23-50 days); 5) NA (34-56 days).

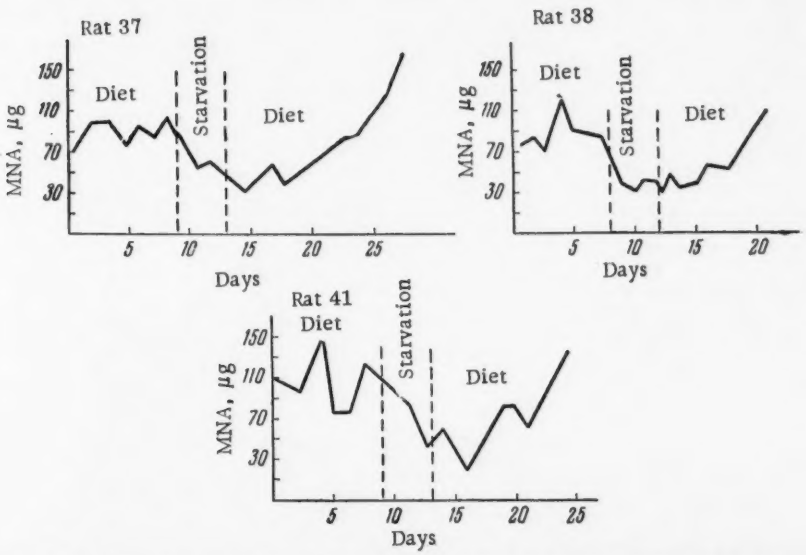

Fig. 2. Excretion of N'-nicotinamide in the urine during starvation (in days).

When a dose of NAA was given to rats in protein starvation more MNA was excreted in the urine than after such a dose in the preliminary period when the diet contained protein; we also found that after a dose of NA, less MNA was excreted than after a dose of NAA. Thus, for example, when the diet of rat No. 21 contained protein, in three days after a dose of NAA it excreted 2620 μg of MNA; after doses of NAA and NA given on the 38th and 43rd day of protein starvation it excreted in the same period 4543 and 1368 μg of MNA respectively, and after doses of NAA and NA on the 50th and 56th day of protein starvation, 9490 and 2470 μg of MNA. An analogous picture was found for the other four rats (Fig. 1).

Series 2. Complete Starvation

In this experiment we used 8 rats weighing 165-170 g; at first they were given the protein diet for 7-8 days, and then only water for four days. During the period of starvation they lost 13-18% in weight. Then the rats were again given the protein diet for 10 days and during this period they regained their weight. The MNA content of the urine was determined daily. The experiment showed that during the four days of complete starvation the animals continued to excrete MNA in the urine although its content in the urine decreased with the weight. After the starvation period the MNA content of the urine gradually increased to the starting level and sometimes even above it.

Fig. 2. shows the curves of daily excretion of MNA for three rats of series 2.

DISCUSSION

The experiments showed that the process of biological methylation of NA can occur not only with methyl groups from dietary methionine, but also with methyl groups formed by endogenous reactions: MNA excretion occurs during protein (and thus methyl group) starvation, and when the animal is completely deprived of food. Endogenous methyl groups can produce methylation even when increased doses of NA or NAA are supplied.

Our previous experiments [8] showed that the ability to carry out endogenous methylation of niacin under certain conditions could occur in those animals which under the usual physiological conditions did not produce MNA and in which niacin metabolism followed another path. Thus, while under ordinary conditions of full nutrition the urine of guinea pigs did not contain MNA, this methylated compound appeared during starvation.

It is worth mentioning that after adding supplements during starvation, more MNA is excreted than with protein in the diet. This agrees with the results of Sarrett and Perlzweig [9] and of Tikhomirova [10] who found increased excretion of MNA in rats with protein deficiencies (4.5% protein).

It is also worth mentioning that after adding NA the rat excretes much less MNA than after adding NAA. This may be because of suppression of NA amidation in protein insufficiency; this process precedes the methylation. It is known that in protein starvation deamination and transamination are also disturbed. At the same time, our experiments show that neither protein nor complete starvation injure the functions of the enzyme system which methylates nicotinic acid.

SUMMARY

In rats whose diet does not contain protein, the ability to methylate nicotinic acid (NA) is retained, even when extra amounts are supplied to the organism. NA is excreted in the urine in the form of N'-methylnicotinamide.

The ability to methylate nicotinic acid is also retained by the organism in full starvation.

An endogenous source of methyl groups can be used by rats for the biosynthesis of N'-methylnicotinamide.

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METABOLISM IN HUMAN THROMBOCYTES

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A number of publications has appeared in recent years dealing with the chemistry of platelets. The majority of the investigations are, however, concerned with the chemical composition of platelets but not with their metabolism.

The presence of the following has been demonstrated in platelets: peptidase, acid and alkaline phosphatases [1]; oxidase and dehydrogenase [2]; monophosphatase, adenosinetriphosphatase, esterase, specific cholinesterase [3]; catalase, amylase, acid pyrophosphatase, glucuronidase, histaminase, lecithinase [4]. It has been established that thrombocytes contain ribonucleic acid (RNA), phosphoproteins (PhP), traces of desoxyribonucleic acid (DNA) [5], the amount of RNA and phospholipids (PhL) in the platelets being the same as in leukocytes [3]. Cytochemical data reveal that thrombocytes contain glycogen, mucopolysaccharides [6], adrenaline [7]. Tulles [8] did not find any glucose utilization or formation of lactic acid in thrombocytes. Campbell et al. [2] observed, on the other hand, O_2 absorption and glycolytic cleavage of sugar down to lactic acid in blood platelets. Morita and Asada [3] demonstrated quantitatively thrombocyte uptake of P^{32} into their ATP, ADP and other organic phosphates.

The question as to whether platelets are living cells is answered by many investigators in the negative. Tulles [8] considers that there is no convincing evidence of metabolic activity of thrombocytes. Maupin [4, 9] regards platelets as cellular fragments possessing a high degree of autonomy, with their own enzymes and respiration. This author, similarly to Tulles, classes thrombocytes as "incomplete cells."

In view of the important physiological role of platelets in the organism of man and mammals, the questions of the biological nature, metabolic activity and chemico-structural independence of thrombocytes are of great interest and practical significance. The present work is devoted to these questions.

METHODS

Experiments were carried out on thrombocytes from healthy subjects (donors). The purity of the platelet suspensions was high: erythrocytes were absent and the leukocyte content was 1 : 50 - 150,000 thrombocytes, in most experiments it was 1 : 100,000. Silicon vessels were used for the isolation and incubation of thrombocytes. A suspension of isolated thrombocytes in serum diluted 1 : 1 with physiologic solution and free of glucose was incubated with various additives at 37° in Warburg apparatus vessels. Usually about 0.3 ml thrombocytes was taken, corresponding to $1.4 \cdot 10^{10}$ platelets. The following determinations were made: O_2 absorption by the suspensions, formation of lactic acid, using the reaction with p-hydroxydiphenyl, P^{32} exchange in various organic phosphorus compounds. Charcoal adsorption method was used [10] for ATP determination. Fractionation of acid-insoluble phosphorus-containing compounds was effected by the Schmidt-Thannhauser method. C^{14} uptake by thrombocyte protein and nucleic acids in the course of metabolic changes of uniformly labeled C^{14}-glucose was studied. For this purpose the platelets were separated from the medium by centrifuging after incubation with C^{14}-glucose, washed with physiological solution and treated with trichloroacetic acid (TCA). The residue was washed once with 10% solution and four times with 5% solution of TCA after which it was extracted for 15 minutes at 90° with 5% TCA. The precipitate was washed once with 5% TCA, once with absolute ethyl alcohol and extracted three times at 65° for 5 minutes with a mixture of ethyl alcohol and ethyl ether (3 : 1). The protein was then washed twice with absolute ethyl ether and applied to aluminum plates from a mixture ethyl ether- petroleum ether (1 : 3) [11]. Corrections were made for autoabsorption. Radioactivity (C^{14}) of nucleic acids was determined in neutralized TCA extracts.

RESULTS

The results of several typical experiments on respiration, glycolysis, phosphorylation as well as on the quantitative content of various phosphorus-containing compounds in thrombocytes are presented in the Table.

Respiration, Glycolysis, Conjugated Phosphorylation and Quantitative Content of Various Organic Phosphorus Compounds in Human Thrombocytes

Composition of samples	Respiration in µl 02	Glycolysis in mg lactic acid	ATP		RNA		PhP		PhL	
			µg, P	RSA	µg, P	RSA	µg, P	RSA	µg, P	RSA
Thrombocytes (T)	761	2.04	67	35	54	30				
T + glucose, aerobic	510	5.9	141	46	70	26				
The same, anaerobic	—	10.2	181	34	75	31				
Thrombocytes (T)	574	1.96	112	24	45	25			645	1.4
T + glucose aerobic	455	8.2	196	23	61	36			835	0.9
The same, anaerobic	—	10.2	192	25	39	26			605	0.9
Thrombocytes (T)	577	1.9	187	25	28	33	9	8.3		
T + glucose, aerobic	434	5.9	238	30	48	27	8	16.0		
The same, anaerobic	—	9.4	239	28	38	41	14	10.0		
Thrombocytes (T)	609	1.9	92	30	44	32	9.5	20	500	1.9
T + glucose, aerobic	434	5.15	130	37	44	27	12.0	12	560	1.4
The same, anaerobic	—	8.65	173	32	48	20	13.0	16	640	1.0
Thrombocytes (T)	392	3.0	148	24	35	33	8.5	21	476	1.3
T + glucose aerobic	332	7.6	177	28	56	28	4.5	46	488	1.6
The same, anaerobic	—	10.8	164	35	35	30	5.5	33	584	1.1
Thrombocytes (T)	405	1.89	76	22	59	14	9.5	6	548	0.62
T + glucose aerobic	314	4.32	75	38	76	13	10.5	9.6	560	0.72
The same, anaerobic	—	6.77	128	25	50	10	13.5	8.7	564	0.47
Thrombocytes (T)	780	3.0	89	32	55	22	—	—	1182	0.74
T + glucose, aerobic	705	7.3	192	39	63	29	12.5	13.5	1015	1.1
The same, anaerobic	—	11.8	234	36	68	27	12.0	18.5	1300	1.4
T + glucose aerobic	492	6.46	171	36	56	28	6.5	23	700	1.9
The same, anaerobic	—	9.24	196	36	56	32	7.5	27	720	1.4
Thrombocytes(T)	560	3.8	117	20	43	29	21	11	—	—
T+ glucose, aerobic	486	5.95	157	37	40	27	20	11	—	—
The same, anaerobic	—	10.7	166	28	63	14	24	12	536	1.1
The same + bromoacetate ($8 \cdot 10^{-4}$ M)	562	0.54	117	17	48	19	20	12	516	2.7
The same + DNP ($8 \cdot 10^{-4}$ M)	468	11.1	117	31	42	17	21	25	460	1.2

Note. Values are given for 1 ml thrombocytes/ 1 hour; RSA – relative specific activity (specific activity of phosphate fraction relative to specific activity of intracellular mineral phosphorus, in %).

The table shows that thrombocytes possess high glycolytic activity with relatively low respiration. Characteristically, respiration does not block glycolysis completely and these two processes coexist in thrombocytes under aerobic conditions. Aerobic glycolysis constitutes about 65% of that possible in anaerobic medium.

Mean indices of metabolic activity of human platelets, based on 1 mg dry weight during 1 hour, were obtained: $Q^{air}_{CO_2} = 12.6$; $Q^{N_2}_{CO_2} = 19.3$; $Q^{glucose}_{O_2} = -3.56$; $Q^{without\ glucose}_{O_2} = -4.4$. It follows from the last two values that thrombocytes exhibit the so-called inverse Pasteur reaction: on addition of the substrate – glucose – platelet respiration diminishes (by an average of 19%). Inhibition of respiration in the presence of sugar may be abolished by addition of monobromoacetate which blocks glycolysis and restores O_2 absorption to the level of the glucose--free sample. This fact indicates that the cause of depression of thrombocyte respiration in the presence of glucose is not glucose itself, i.e., not the substance itself, but the process initiated by its presence, viz., glycolysis. Prevention of glycolysis in thrombocytes by depriving them of sugar or by addition of glycolytic poison to the medium containing sugar increases absorption of O_2.

Platelets exhibit close metabolic similarity to polymorphonuclear leukocytes and cancer cells. This concerns high glycolysis values, relatively low respiration, presence of anaerobic glycolysis and inverted Pasteur reaction. However, the similarity of thrombocytes to the cells mentioned above extends still further and includes the principles and mechanisms of resynthesis of ATP – a substance placed centrally in cellular energy metabolism.

The table shows that platelets contain considerable amounts of ATP. It is very remarkable that in the presence of sugar in the medium the amount of ATP in anaerobic conditions is no less than its content under conditions of aerobiosis (164 ± 11 and 150 ± 4 μg labile ATP phosphorus in 1 ml thrombocytes respectively; data of 16 experiments). This in itself suggests the possibility of complete resynthesis of ATP in thrombocytes under anaerobic conditions. On the other hand, studies of P^{32} turnover in thrombocytes show that the rate of metabolic changes undergone by ATP, judging by relative specific activity (RSA) of its labile phosphorus, is practically identical under aerobic (average 35%) and anaerobic (31%) conditions. Consequently, radiochemical data also lend support to the view that the energy metabolism of thrombocytes is equally complete under aerobic and anaerobic conditions. This property was noted earlier by us with respect to cancer cells [12] and leukocytes [13]. Evidently, the ability to synthesize ATP equally effectively under both anaerobic and aerobic conditions is common to all cells with high levels of glycolytic activity and relatively feeble respiration.

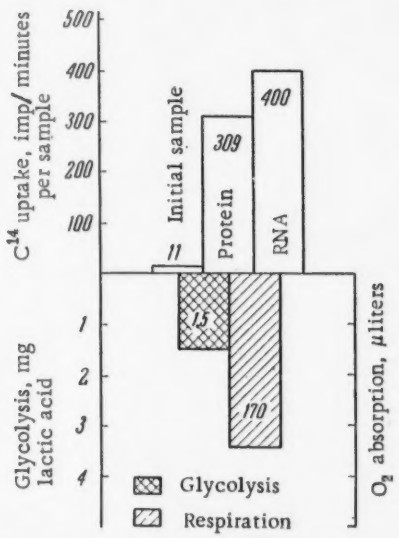

Fig. 1. C^{14} uptake into protein and ribonucleic acid of human thrombocytes by way of C^{14}-glucose utilization: Composition of samples: 0.2 ml thrombocytes (20 mg protein) + C^{14} glucose 2.25 mg (96000 imp/ minute). Medium serum + physiological solution (1 : 1). Indubation 100 minutes at 37°. Initial sample – sample which, unlike the experimental sample, was kept at 0° for 100 minutes (control with respect to complete removal of C^{14} by washing).

In the absence of sugar from the medium, even with free access of air, considerable decline in ATP content of platelets is observed, as well as an appreciable decrease in the rate of renewal of its labile phosphorus.

Monobromoacetate in the presence of glucose stimulated thrombocyte respiration with slowing of ATP phosphorus turnover and decrease in the amount of this compound. It must be noted that disturbances of phosphorus metabolism caused by monobromoacetate are much less marked in thrombocytes than in leukocytes and cancer cells. In the case of human granulocytes and mouse ascitic cancer cells bromoacetate, in the presence of glucose in the medium, acts not only on glycolysis and phosphorylation coupled with it but also on esterification associated with respiration [14, 15]. In the case of human platelets this glycolytic poison lowers ATP content and rate of renewal of its labile phosphorus only to the level of glucose-free samples without, evidently, affecting phosphorylation coupled with oxidative processes. The manifestations of the bromoacetate effect are unequal in different cells. The main effect – arrest of glycolysis and phosphorylation coupled with it – may be accompanied by a number of side effects if respiration is maintained; these effects are determined on the one hand by the presence of sugar in the medium and, possibly, reserves of glycogen in the cell, and on the other hand by the interrelation of activities of separate enzyme systems, the most important of these apparently being enzymes responsible for re-esterification and mineralization of organic phosphates, most especially ATP. Owing to this the effect of bromoacetate on the energy metabolism of even such closely related, metabolically, cells as cancer cells, leukocytes and thrombocytes shows variation.

The "uncoupling" poison 2 : 4-dinitrophenol (DNP) increased the rate of aerobic glycolysis and had relatively little effect on the quantitative content and rate of turnover of labile ATP phosphorus. The inability of DNP to produce sharp changes in ATP resynthesis could be explained by its impairing respiratory phosphorylation in thrombocytes and complete abolition of the Pasteur effect, thus enhancing aerobic glycolysis to the level of anaerobic

glycolysis. This leads to increased esterification of phosphorus associated with glycolysis which compensates the phosphorylation deficit produced by "uncoupling" in the respiratory cycle.

Judging by the mineral phosphorus turnover the penetration of substances from the medium into the platelets is not a simple matter of physicochemical diffusion but an active biological process. Radioactive mineral phosphorus practically did not penetrate into the thrombocytes at 0°, but radioactivity within the platelets increased progressively at 37° when respiration and glycolysis were taking place. It is therefore possible to speak of active transport of substances from the external environment into the thrombocytes coupled with metabolism, and of the presence of at least a functional membrane in the platelets.

Investigation of thrombocytes for DNA content gave negative results. This is associated with the absence of a nucleus or its biological equivalent in platelets. But thrombocytes contain appreciable amounts of RNA. The amount of RNA phosphorus constitutes on average 57 μg/ ml thrombocytes which is about one-quarter of RNA content in normal leukocytes. The renewal of this nucleic acid phosphorus is much more rapid in platelets than in leukocytes. The RSA of thrombocytes, on incubation for one hour, amounts to an average, deduced from all the experiments, of 31% with respect to specific radioactivity of mineral phosphorus and reaches the RSA of ATP phosphorus. If, however, the RSA of RNA phosphorus is calculated with respect to the specific activity of phosphorus of the immediate precursor, i.e., ATP, the figure proves to be still higher – 73%. High metabolic indices are also found for PhP whose amount in platelets is not large. The high metabolism in RNA and PhP suggests high functional activity of these compounds in thrombocytes.

The PhL fraction in platelets differs little from the corresponding fraction in normal leukocytes with respect to quantitative composition and metabolic rate.

Experiments with C^{14}-glucose were of theoretical significance. They permitted the tracing not only of the energy conversions of this substrate but also the participation of the products of its metabolism in the plastic metabolism of thrombocytes. Addition of uniformly lableled C^{14}-glucose to platelets led to the appearance of labeled carbon C^{14} in RNA protein in addition to its conversion to lactic acid and CO_2.

Approximately 0.3% of the radioactivity of the utilized C^{14}-glucose appeared in the protein (Fig. 1). Consequently, cleavage of labeled sugar in the course of glycolysis and respiration in thrombocytes is accompanied by effective uptake of C^{14}-particles into the RNA proteins by way of the intermediate products of carbohydrate breakdown which are formed in this process.

The fact of "renewal" of the carbon skeleton of protein in RNA of platelets may serve as one of the criteria of manifestations of life and metabolic activity, and in conjunction with data concerning complete energy metabolism it points to chemico-structural and metabolic-physiological independence and autonomy of thrombocytes.

DISCUSSION OR RESULTS

The metabolic coefficients obtained by us for human thrombocytes ($Q_{O_2}^{\text{glucose}} = 3.56$; $Q_{O_2}^{\text{without glucose}} = 4.4$; $Q_{CO_2}^{\text{air}} = 12.6$; $Q_{CO_2}^{N_2} = 19.3$) are very close to the characteristics considered by Warburg as specific for cancer cells.

Values for respiration and glycolysis of just such order had been found in Warburg's laboratory for many malignant tumors [16]. Among new data the metabolic indices cited by Warburg [17] for the low malignant cancer cells of Earle may be mentioned: $Q_{O_2} = 13$; $Q_{CO_2}^{O_2} = 10$; $Q_{CO_2}^{N_2} = 25$. The relation of absolute values of glycolysis and respiration in these cells is seen to be much less characteristic for malignant cells than is the case in thrombocytes. At the same time Warburg attributes great significance to the ratio $Q_{CO_2}^{N_2} / Q_{O_2}$ with respect to characterization of cancer metabolism since, according to his point of view, increase of glycolytic activity and decrease of respiration are characteristic of cancer cells; it is not simply weak respiration that is essential in cancer metabolism but respiration which is low compared to glycolysis. For cells taken by Warburg as a classic example of malignant tumor, viz., mouse ascitic cancer cells, we [12] and later Warburg [18] found the following respiratory and glycolytic indices: $Q_{O_2} = 5\text{-}10$; $Q_{CO_2}^{N_2} = 50\text{-}60$. The ratio $Q_{CO_2}^{N_2} / Q_{O_2}$ can be in this case from 5 to 12. The ratio $Q_{CO_2}^{N_2} / Q_{O_2}$ in platelets is 5.4. Exactly the same figure – 5.4 – was obtained by us for human leukocytes also $Q_{CO_2}^{N_2} = 28.9$; $Q_{O_2} = 5.4$.

The values mentioned cause serious doubts concerning the specificity for cancer cells of those quantitative ratios of respiration and glycolysis noted by Warburg with respect to these cells. The presence of aerobic glycolysis and inverted Pasteur reaction also now appear to be nonspecific for malignant tumors since both these phenomena were observed by us in leukocytes and thrombocytes.

Hence aerobic glycolysis, high anaerobic glycolysis, inverse Pasteur reaction, low – both absolutely and relatively to glycolysis – respiration are also characteristic for some noncancerous cells.

In this light, data on resynthesis of ATP in platelets also acquire interest. The table shows that the quantitative content of glucose is practically identical under aerobic and anaerobic conditions. It may be concluded from this that ATP resynthesis under anaerobic conditions is as complete as in the presence of atmospheric O_2. It is difficult to indicate any other cells of the animal organism, apart from leukocytes, which have this property of maintaining normal energy metabolism equally effectively under aerobic and anaerobic conditions. We noted this remarkable ability earlier in cancer cells [12, 15] and these cells appeared to be unique in this sense. Now we must admit that complete ATP resynthesis under aerobic and anaerobic conditions is also a property of a number of noncancerous cells.

Consequently, the effective resynthesis of ATP under aerobic and anaerobic conditions, together with low respiration, high aerobic and anaerobic glycolysis, inverse Pasteur reaction, is a metabolic feature common to cancer cells, certain types of leukocytes, thrombocytes and spermatozoa [19]. It appears impossible, in the light of these data, to regard these features taken as a whole as specific for malignancy or as being causally related to malignant processes. Evidently the manifestation of these distinctive features of metabolism is associated not with a pathologic state but with some essential role in biology and cell function, possessing something in common in the widest physiological sense (functional stress load and so forth).

Another aspect of the results of this investigation is concerned with characterization of thrombocytes as independent chemico-structural formations with metabolic properties of a cell. The active transport of substances into platelets, associated with metabolic processes; complete respiration and glycolysis coupled with resynthesis of ATP; renewal of protein and RNA; presence of direct and inverse Pasteur reactions – all these properties belong to complete cells but had never been demonstrated in all their complex totality in noncellular structures. It must be noted that the mechanism of the Pasteur effect is extremely characteristic for biological formations of cellular type. The natural Pasteur effect is observed only in whole, undamaged cells and any disintegration which disrupts cellular unity leads to disorganization of metabolism and interrelations of respiration and glycolysis and hence disappearance of the Pasteur effect. Imitation of the Pasteur reaction in subcellular systems is achieved by the use of a number of artificial agents and procedures [19, 20].

It was shown in the experimental part of the work that the high anaerobic glycolysis exhibited by thrombocytes was greatly inhibited (by about 35%) in the presence of air, which suggested that the Pasteur mechanism was at work in platelets. This fact, together with the inverse Pasteur reaction shown by thrombocytes, indicates that thrombocytes have a cellular type of structural organization.

Thus, despite the absence of a nucleus (which holds for other animal cells as well, e.g., mammalian erythrocytes), platelets can be characterized biochemically as biologic formations possessing the main qualities of a cell.

SUMMARY

Human platelets possess high metabolic acitivity. 1 ml fresh thrombocytes ($4.7 \cdot 10^{10}$ platelets) absorbs during 1 hour at 37° in serum, 445 μliter/O_2 in the presence of glucose ($Q_{O_2}^{\text{glucose}} = 3.56$) and 551 μliter/O_2 ($Q_{O_2}^{\text{without glucose}} = 4.4$) in the absence of sugar. Inhibition of respiration (by about 20%) on addition of the carbohydrate attests to the presence of the so-called inverse Pasteur reaction in thrombocytes.

1 ml platelets forms in 1 hour in anaerobic medium 9.7 mg lactic acid ($Q_{CO_2}^{N} = 19.3$) and 6.3 mg lactic acid under aerobic conditions ($Q_{CO_2}^{\text{air}} = 12.6$).

Considerable amounts of ATP have been found in thrombocytes, viz., 150 ± 4 μg/ml platelets. In the presence of sugar in the medium ATP does not decrease under anaerobic conditions – 164 ± 11 μg/ml thrombocytes. On incubation for 1 hour in serum at 37° in the presence of glucose the relative specific radioactivity of labile ATP phosphorus reached 35% under aerobic conditions and 31% under anaerobic conditions.

The platelets were found to have: RNA phosphorus 57 ± 2.9 μg; phosphoprotein phosphorus (PhP) 9.2 ± ± 1.5 μg; phospholipid phosphorus (PhL) 676 ± 42 μg/ml fresh thrombocytes. No DNA was found.

RNA and PhP phosphorus is renewed in thrombocytes very rapidly, only a little less so than in the case of metabolism of labile ATP phosphorus. Phosphorus exchange in PhL is slow.

It was shown with the help of uniformly labeled C^{14}-glucose that there is renewal of the carbon skeleton of protein and RNA in thrombocytes at the expense of C^{14}-fragments of glucose utilized in the process of glycolysis and respiration.

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A STUDY OF THE BIOGENESIS OF POTATO GLYCOALKALOIDS BY THE METHOD OF LABELED ATOMS

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There are two basic glycoalkaloids in the potato, closely related in structure: solanine and chaconine. They contain the same aglycone, solanidine, a steroid alkaloid, but the sugar portion, a branched trisaccharide, contains galactose, glucose, and rhamnose in solanine [1], and glucose and two rhamnoses in chaconine [2].

The chemical structure of the glycoalkaloids has now been well studied, but little is known of the biosynthesis and reactions of these substances in the plant. It is therefore interesting to study the formation and reactions of the potato glycoalkaloids by the method of labeled atoms.

EXPERIMENTAL

Preparing Radioactive Potato Glycoalkaloids

The experiments were performed with sprouts of "Berlikhingen" type of potatoes, since the sprouts contain the greatest amount of glycoalkaloids. The absence of chlorophyll in the sprouts made the separation and purification easy.

Batches of sprouts containing about 20 g of sprouts for each experiment were made up to be as homogeneous as possible. The sprouts were supported on paraffin gauze on a Koch flask. In each flask was placed 100 ml of 0.2 N Knop solution which contained 20 μC of $KCH_3C^{14}O_2$ with pH 6.5-6.8. The experiments were carried out at 20-25°. After definite time intervals samples of sprouts were taken, carefully washed with water, fixed, air dried, and ground to a powder. The dry powder was spread in a thin layer on a disk and the radioactivity was determined with a counter. At the same time the radioactivity was determined for the nutrient Knop solution on which the sprouts had been placed.

TABLE 1

Uptake of C^{14} Acetate by Potato Sprouts

Duration of the experiment in days	Intensity calculated for the sprouts in counts/ min/ mg of dry substance	Radioactivity of the Knop solution in μC per 100 ml of solution
0	0	20
1	72	5.8
2	161	1.4
3		1.0
6	158	0.7
14	88	0.3

The results are given in Table 1.

Table 1 shows that the uptake of $KCH_3C^{14}O_2$ by potato sprouts occurs very rapidly; even after one day only 29% of the original radioactive acetate remained in the solution.

To determine the radioactive glycoalkaloids, the dry, powdered potato sprouts were treated with boiling ethyl alcohol for 30 min (20 parts of alcohol to 1 part of sprouts), filtered hot, the precipitate again treated with alcohol, and the procedure repeated four or five times. The filtrates were combined and the alcohol distilled off in a vacuum; the residue was dissolved in N HCl and filtered. The filtrate was made alkaline to phenolphthalein with ammonia and heated to 60°; the glycoalkaloids then precipitated. The precipitate was filtered, washed with dilute ammonia

until all chloride ion was removed, and dried.

For further purification the dry precipitate was dissolved in hot alcohol and filtered; the filtrate was evaporated. After removal of the alcohol the glycoalkaloids remained as a white crystalline powder and were dissolved in 50% alcohol which contained 0.5% HCl,* and were deposited on a disk.

The radioactivity of the glycoalkaloid hydrochlorides was measured on a counter. Corrections for internal absorption were not made because the values for the radioactvity were only relative.

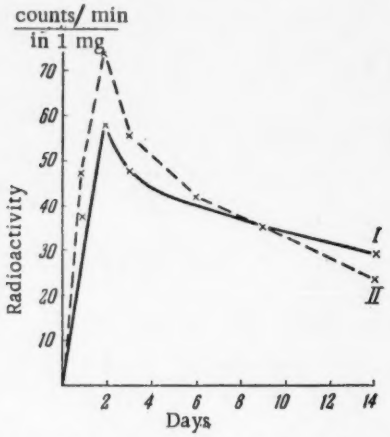

Fig. 1. Radioactivity of Glycoalkaloids from Sprouts.
I) Natural illumination; II) dark.

The purity of the preparation was tested by the color reaction with sulfuric acid and 1% formalin [3].

Experiments with the sprouts were carried out with natural illumination and in the dark. Results are given in Fig. 1.

The experiments showed that the labeled carbon from the $KCH_3C^{14}O_2$ which was absorbed by the sprouts under normal illumination or in the dark was detected in large part in the glycoalkaloids. The maximum radioactivity was found in the glycoalkaloids in two days, after which it fell to 40-45% and then remained unchanged for a long time.

Thus, the potato glycoalkaloids are substances which take part in plant metabolism.

Distribution of Labeled Carbon between the Aglycone and the Sugar Portions of the Glycoalkaloids

It was interesting to determine the distribution of the labeled carbon between the aglycone and the sugar portions in the radioactive glycoalkaloids.

TABLE 2

Intensity Calculated for Glycoalkaloids and Aglycones

In counts/ min in 1 mg of product

Exposure in days	Intensity		Distribution of C^{14} between aglycone and sugar in %		Exposure in days	Intensity		Distribution of C^{14} between aglycone and sugar in %	
	Glycoalkaloids	Solanidine	In aglycone	In sugar		Glycoalkaloids	Solanidine	In aglycone	In sugar
Normal Conditions					Dark				
0.25	0	0	—	—	1	47	56	57.1	42.9
1	37	80	103.7	0	2	72	82	54.6	45.4
2	57	122	105	0	3	55	75	65.5	34.5
3	47	85	86.7	13.3	6	42	52	59.5	40.5
9	40	76	91.1	8.9	14	29	40	66.3	33.7
14	30	53	84.9	15.1					

For hydrolysis the glycoalkaloids were dissolved in alcohol, treated with an equal volume of 2 N H_2SO_4 and refluxed for 6-8 hours. Completeness of hydrolysis was tested by paper chromatography [3]. Hydrolysis in aqueous alcohol prevented dehydration of solanidine into solanthrine [4]. After hydrolysis, the filtrate was made alkaline with ammonia. The precipitated solanidine was filtered and washed carefully until no reaction for SO_4 ion was

* The solution of the glycoalkaloids in aqueous alcohol which contained 0.5% hydrochloric acid deposited well on the disk, and the preparation was evenly distributed over the surface.

given. We were not able to isolate the sugars in pure form after hydrolysis of such small amounts of glycoalkaloids. The radioactivity of the sugar portion was estimated by comparison of the activity of the whole glycoside and that of the aglycone obtained from it.

The solanidine precipitate was dried, dissolved in 50% alcohol which contained 0.5% hydrochloric acid, and was deposited on the disk as the hydrochloride. After evaporation of the solvent, the radioactivity was determined.

The results are given in Table 2.

Table 2 shows that the labeled carbon absorbed by the sprouts was found chiefly in the aglycone part of the glycoalkaloids in the experiments carried out under conditions of normal illumination. Different results were obtained when the experiments were carried out in the dark. In this case the labeled carbon entered the aglycone and the sugar part almost equally. This may occur because in the dark, when photosynthesis is excluded, acetic acid is used in the formation of sugars. The data also confirm once more the great mobility of the glycoalkaloids in plant metabolism.

Significance of the Roots in Synthesis of Potato Glycoalkaloids

Prokoshev and co-workers [5] found by the method of grafting that glycoalkaloids in the potato were formed in the leaves and perhaps in the stem but not in the roots, as distinct from the alkaloids of the nicotine and tropane groups which were formed in the root. Recently Il'in[6] confirmed in studies using labeled atoms that biosynthesis of the nicotine group of alkaloids occurred only in the root.

We decided to use the method of labeled atoms to study the significance of the root in the synthesis of potato glycoalkaloids.

TABLE 3

Uptake of $KCH_3C^{14}O_2$ by the Glycoalkaloids of Potato Grafts and Sprouts

Material	Duration of experiment in days	Intensity calculated in counts/ min/ mg		Distribution of C^{14} between aglycone and sugar in %	
		Glyco-alkaloids	Solani-dine	Aglycone	Sugar
Sprout with root	3	22	38	83.1	16.9
Sprout without root	3	20	34	81.7	18.3
Graft with root	4	15	–	–	–
Graft without root	4	20	–	–	–

The experiments were conducted with potato sprouts and grafts. The lower part of the sprout, which carried the root, was cut off. The top of the shoot was put into Knop solution which contained $KCH_3C^{14}O_2$. Potato grafts were first rooted, then the root was cut off and the rootless graft was put into Knop solution. The course of the experiments and the separation of the radioactive glycoalkaloids and aglycones were described above. We also determined the distribution of the labeled carbon between the aglycone and the sugar portion of the glycoalkaloids prepared from rootless and control sprouts.

The experimental results are given in Table 3.

The experiments showed that both sprouts and grafts with roots removed took up $KCH_3C^{14}O_2$ and part of the labeled carbon was found in the glycoalkaloids; most of the radioactive carbon was found in the aglycone portion, as in the experiments with the sprouts.

SUMMARY

The method of labeled atoms shows that potato sprouts take up acetic acid, and part of the labeled carbon from the radioactive acetic acid is found in the glycoalkaloids.

The greatest radioactivity is found in the glycoalkaloids from sprouts kept two days on radioactive acetic

acid and after this time the radioactivity decreases; from this we conclude that the glycoalkaloids are involved in the metabolism of the potato plant.

The radioactive glycoalkaloids isolated from potato sprouts which were grown experimentally under conditions of normal illumination had the labeled carbon almost entirely in the aglycone portion, the solanidine. The radioactive glycoalkaloids from sprouts grown in the dark contained labeled carbon in both the aglycone and the sugar portions.

We have confirmed by the method of labeled atoms that the glycoalkaloids can be formed in potato grafts and sprouts without participation of the roots.

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PHOSPHORUS METABOLISM IN THE EMBRYONIC DEVELOPMENT OF STURGEON

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The embryonic development of sturgeon has been studied very thoroughly from the morphological standpoint [1] but the physiology and biochemistry of the development of these fish has not yet been studied adequately.

Considering the extremely important role of phosphorus-containing compounds in the metabolism of developing embryos, we first of all turned to the study of this aspect of the biochemistry of embryonic development of sturgeons. In the present work the results of determinations of the content of the main fractions of phosphorus-containing compounds and their metabolism in the developing ova of sturgeon – Acipenser stellatus Pall and Huso huso L. – are reported.

MATERIALS AND METHOD

At present fertilized roe of sturgeons can be obtained only from artificial hatcheries. Our material was obtained from the sturgeon hatchery Azdonrybvod at Rogozhkino (Rostov region). The roe, at different developmental stages, was taken from the commercial incubation installations and fixed for analyses, which were performed in Moscow.

The inadequacy of a purely analytical approach, which seemed the only possible one under these conditions, became apparent when it became clear that the content of most of the phosphorus compound fractions showed little change in the process of development. This gave rise to the idea of using the isotopic method along with the analytical one.

Introduction of appreciable amounts of P^{32} into intact mature ova by means of incubating the latter in solutions containing the isotope proved impracticable owing to the very slight permeability of ovum membrane to ions. Under our conditions, the most convenient method was found to be that of introducing P^{32} into the ova by way of the female's body. This method is simple and ensures adequate P^{32} uptake by the phosphorus compounds in the ova, but it has a substantial disadvantage: between the moment of administration of the isotope to the female sturgeon and the taking of samples of developing roe at late developmental stages the isotope has time to be so redistributed between the main fractions that the interpretation of the relations between the fractions becomes difficult. However, during the first half of the development this method gives sufficiently good results.

P^{32} was administered to a 6-kg female sturgeon intramuscularly in the form of a neutral solution of $Na_2HP^{32}O_4$ with a total activity of 1.3 mc, after which an injection of pituitary gonadotropic hormone was made. Ovulation occurred after 35 hours and the female sturgeon was sacrificed. Mature ova were taken by opening the abdominal cavity, fertilized with sturgeon sperm and freed from viscid material by means of a slit suspension. Development of the ova took place in the Iushchenko [2] type of incubator and reached the stage of larval hatching without any appreciable deviations from the normal.

Roe samples were taken from the beginning of development to stage 33 (according to the Detlaf and Ginzburg [1] classification); a total of 11 samples was taken. In the case of Huso huso the material was also fixed at

stages 35 (before hatching) and 36 (just hatched larvae).

Roe samples were fixed by four-five volumes of boiling 95% ethyl alcohol which preserved the composition of the roe adequately as seen from comparison of various methods of fixation.

In this material the phosphorus content, and in the case of sevriuga (Acipenser stellatus Pall) to which the isotope had been administered, radioactivity as well, was assayed in the following fractions: acid-soluble in which mineral and acid-labile phosphorus (7'-P) were determined separately, phospholipids (PhL), phosphoproteins(PhP), ribonucleic acid (RNA) and desoxyribonucleic acid (DNA).

Phosphorus content was also checked in the protein residue after extraction of all the phosphorus-containing fractions.

The fractionation was based on the combined methods of Schmidt-Thannhauser and Schneider in the form described by Volkin and Cohn [3]. Fractionation was performed on 10 ova in the case of beluga (Huso huso) and 20 ova in the case of sevriuga. Five times this amount of the material was taken for determinations of DNA which is present in relatively small amounts in the roe; the DNA fraction was isolated by a simplified procedure as follows.

Homogenized material was freed from fatty substances, then treated with 1 N KOH at 37° for 20 hours without preliminary extraction of low-molecular components with acid, since acid treatment could lead to certain alkali-lability of DNA [3]. On termination of hydrolysis the mixture was neutralized with 6 N $HClO_4$ with cooling, then DNA and protein were precipitated from the hydrolyzate by addition of $HClO_4$ to a concentration of 2.5%. The hydrolyzate was discarded and the precipitate, washed several times in a centrifuge with 5% $HClO_4$, was hydrolyzed with 5% $HClO_4$ at 90° for 15 minutes. The protein precipitate was washed several times, with centrifuging, until complete extraction of DNA hydrolysis products was effected. The combined centrifugates were analyzed for DNA.

In view of the high lipid content of the roe, which made its treatment difficult, removal of fatty material was carried out prior to acid extraction. The transposition of these two operations did not distort the results: analyses of the acid-soluble fraction obtained directly from the live material gave the same quantities of mineral and 7'-P as those obtained after preliminary "defatting" (Fig. 4).

Phospholipids were extracted by refluxing 125 alcohol-fixed ova with a further 30 ml boiling alcohol for one hour. This procedure was repeated three times to ensure complete extraction of PhL. From alcohol in which the roe was fixed an aliquot sample corresponding to 125 ova was withdrawn and added to the combined alcoholic extracts. Total phosphorus was determined in part of the alcoholic extract; this phosphorus corresponded to the sum of PhL phosphorus and that part of the acid-soluble fraction which passed into the alcohol. Determination of acid-soluble phosphorus in the alcoholic extract was carried out on the dry residue of the latter dissolved in butanol, the butanol solution being extracted with 5% $HClO_4$.

Combustion of samples for the determination of total phosphorus in all fractions was carried out in a thermostatically controlled muffle at 150°, using perchloric acid as catalyst according to the method of Bessey, Lowry and Brock [4].

Colorimetric determination of phosphate was based on the method of Fiske and Subbarow [5] except that eikonogen was replaced by 1% solution of ascorbic acid as recommended by Lowry and Lopez [6]. Determination of optic density was made with the help of an FEK-M photoelectric colorimeter.

In order to estimate inorganic phosphate the blue phosphomolybdenum complex was taken up in isobutanol [7]. This procedure is expedient both from the point of view of removing errors associated with contamination of inorganic phosphate precipitated by the Dellory [8] method with radioactive phosphorus from other fractions [7] and from the viewpoint of those cases when addition of molybdate to the solution being analyzed causes separation of a precipitate, since this precipitate removes with it a certain part of inorganic phosphate. Extraction of color from the samples being studied was always effected by the same volume of isobutanol (2.5 ml).

In addition to phosphorus determination in the fraction RNA + PhP, ribose was also estimated, using a modified Meybaum method [9], and in the DNA fraction desoxyribose was estimated by the Ceriotti method [10]. The intensity of these color reactions was determined at 660 and 490 mμ respectively, using a SF-11 spectrophotometer. In DNA determinations the calculations were made with respect to a standard solution of DNA from calf thymus.

DNA was also determined in sevriuga sperm. The sperm was washed three times to remove seminal plasma; this was achieved by repeated dispersion in 0.1 M solution NaCl and centrifuging. The number of sperms was de-

termined by counting in a Goriaev chamber. A suspension of washed spermatozoa amounting to 0.5 ml whole sperm was treated similarly to the description given above for DNA determination in the roe, with the difference that no "defatting" was performed and the alkaline hydrolysis was carried out in the presence of 0.33 N KOH. Phosphorus content, desoxyribose by the Dische method [11], and absorption in ultraviolet at 255 mμ were determined in the DNA fraction.

Radioactivity of the fractions was measured with the help of a Type B counter installation with aluminum tube AC-2 with count effectivity of about 20%. Radioactivity of the fractions was determined in the dry residues of aliquots of these fractions. The radioactivity of inorganic P in the acid-soluble fraction and the RNA + PhP fraction was determined on the dry residue of the isobutanol extract.

RESULTS

Determinations of the total phosphorus content of beluga and sevriuga ova showed that it remained unchanged throughout the period studied and constituted on an average 88-89 μg and about 42 μg per ovum respectively.

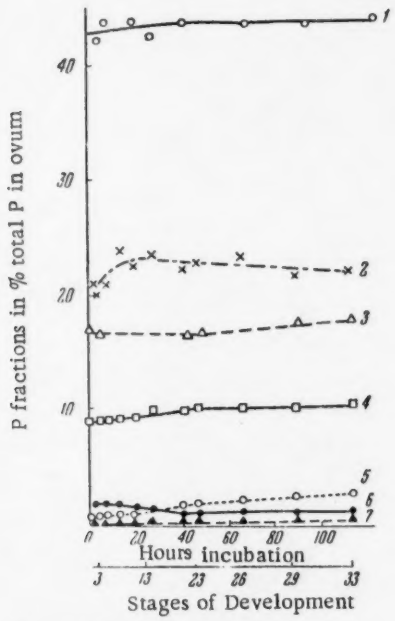

Fig. 1. Phosphorus content of different fractions in % of total phosphorus of sevriuga ova at different stages of development. 1) PhP; 2) PhL; 3) RNA; 4) total P of acid-soluble fraction; 5) inorganic P; 6) 7-minute P; 7) DNA.

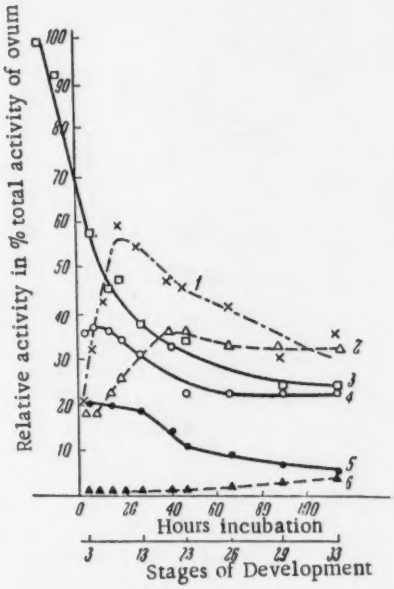

Fig. 2. Change in relative phosphorus activity in separate fractions in the course of development of sevriuga ova. 1) PhL; 2) RNA; 3) total P of acid-soluble fraction; 4) inorganic P; 5) 7-minute P; 6) DNA.

It was also found that the total amount of P^{32} in sevriuga ova, from the beginning to the end of the period studied, remained practically the same and corresponded to an average of 15,600 imp/ minute per 100 ova. We thought it expedient to express the activity of the separate fractions in units of relative activity, taking total radioactivity contained in the ovum as 100%. The radioactivity of the fractions was also expressed in units of specific acitivity (SA) which was calculated by means of dividing the value of activity of the fraction in imp/ minute by the amount of μg phosphorus of this fraction.

Figure 1 shows data on the relative content of different fractions of phosphorus-containing compounds at different points in the embryonic development of sevriuga, starting with the fertilized ovum and ending with the period of active movements of the embryo within the membranes.

It can be seen from Fig. 1 that the absolute quantities of phosphorus in different fractions change insignificantly during development. However, Figs. 2 and 3 show that redistribution of radioactive phosphorus between these fractions takes place, which indicates the existence of intense phosphorus exchange in the process of development.

For detailed consideration of the phosphorus metabolism of developing ova of sturgeons, data are presented below with reference to each fraction separately.

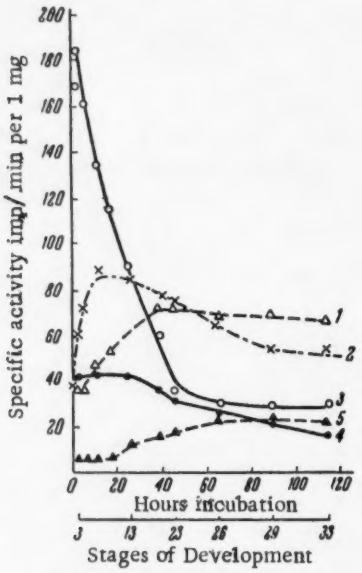

Fig. 3. Change in specific activity of phosphorus in different fractions during development of sevriuga ova. 1) RNA; 2) PhL; 3) inorganic P; 4) 7-minute P; 5) DNA.

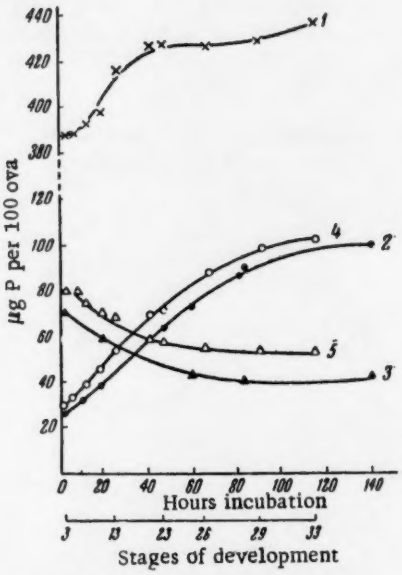

Fig. 4. Change in content of total, inorganic and labile phosphorus in acid-soluble fraction during development of sevriuga ova. 1) Total P; 2) and 3) inorganic and 7-minute P respectively, from roe fixed with hot alcohol; 4) and 5) the same from fresh roe.

Mineral and 7-minute P. Analyses of the acid-soluble fraction revealed that its total content of phosphorus amounted to an average of 3.8 µg or 9% of total phosphorus in the unfertilized ovum. As can be seen from Fig. 2, the amount of total phosphorus of this fraction increases slightly in the course of development, reaching an average of 4.5 µg at stage 33 (10.7%).

Relatively little of total phosphorus of this fraction appears as inorganic and 7-minute P. The unfertilized sevriuga ovum contains 0.3 µg inorganic and 0.8 µg labile phosphorus which corresponds to 0.7 and 1.9% of the phosphorus in the ovum respectively. The labile phosphorus content drops gradually with development, whereas inorganic phosphorus shows an increase (Fig. 4). Similar changes take place in beluga roe. These data, obtained from fixed material, were confirmed by analyses of the acid-soluble fraction obtained directly from fresh material.

We succeeded in tracing the uptake of P^{32} by the acid-soluble fraction of sevriuga ova as early as the period preceding ovulation. It was found that the isotope penetrated the ova very rapidly, being taken up first of all by the acid-soluble fraction. 17 hours after injection practically all the P^{32} contained in the ovum appears in this fraction. Later the P^{32} concentration in this fraction decreases as the result of exchange with other fractions, but by the time of taking samples of ovulated roe about 60% P^{32} still remains in the acid-soluble fraction (Figs. 5 and 6).

After fertilization the relative and specific activities of the acid-soluble fraction fall, but even at late stages of development the latter contains 25% of total activity (Figs. 5 and 6).

Change in the relative activity of inorganic and 7-minutes P is illustrated in Fig. 5. Attention is drawn to

the marked acceleration of P^{32} loss from these fractions during the period between 20 and 30 hours of incubation which corresponds roughly to the initial stages of gastrulation.

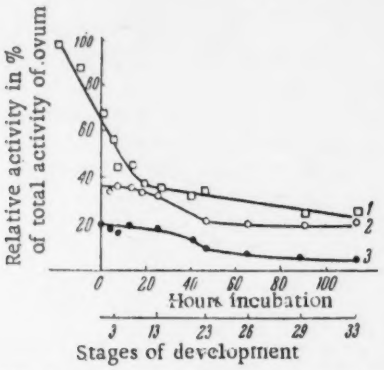

Fig. 5. Relative activity of inorganic, 7-minute and total phosphorus in the acid-soluble fraction of developing sevriuga ova.
1) Total; 2) inorganic; 3) 7-minute.

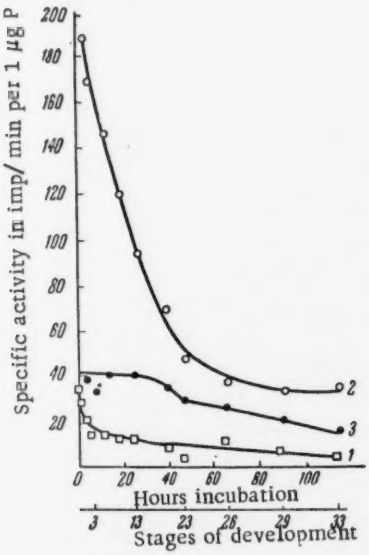

Fig. 6. Specific activity of inorganic, 7-minute and total phosphorus in the acid-soluble fraction of developing sevriuga ova.
1) Total; 2) inorganic; 3) 7-minute.

Comparison of the SA of inorganic and labile phosphate reveals substantial differences between them at the beginning of the development of ova (Fig. 6). Inorganic P during the first hours of development exceeds all other fractions with respect to SA. During subsequent hours the SA of inorganic phosphorus drops rapidly until the end of neurula formation, after which it remains on the same level. The SA of labile phosphorus remains unchanged until the onset of gastrulation after which it begins to decline, evidently in connection with increased exchange of high-energy phosphate bonds. The closely approaching values of the SA for inorganic and 7-minute phosphorus during the second half of incubation of the ova show the presence of intensive exchange between these fractions. By contrast, the sharp difference between them observed prior to gastrulation creates the impression that during this period the exchange between inorganic and labile P is not great.

Phospholipids. Sturgeon roe is extremely rich in PhL. One beluga ovum contains an average of 20 µg PhL phosphorus or about 22% of the total phosphorus, while a sevriuga ovum contains an average of 9.5 µg (about 23%) PhL phosphorus. During development the P content of this fraction remains practically unchanged, showing only a slight increase at the beginning of development (Fig. 7). Nonetheless, the intensive uptake of P^{32} into the PhL observed from the first hours of development and subsequent drop in activity indicate extremely energetic renewal of PhL phosphorus during the period studied. The fall in PhL fraction activity after 20 hours of incubation is explained by exchange between it and other fractions (first of all, probably, with the acid-soluble one) which by this time have lower activity. This is illustrated well in Figs. 2 and 3.

Phosphoproteins. The most important fraction with regard to phosphorus content is the PhP fraction. Thus, in the sevriuga ovum the average PhP phosphorus content is 18 µg, i.e., about 44% of the total phosphorus in the ovum. Beluga roe is equally rich in this form of phosphorus – 40 µg per ovum (about 45%). Phosphoproteins are localized mainly in the yolk. Even at the hatching stage the yolk includes over 80% of all the embryonic phosphoproteins.

The PhP phosphorus content in sevriuga roe is practically unchanged during the course of development (Fig. 1); the same applies to beluga roe although we expected to find a decrease in phosphorus of this fraction. It may be that this decrease failed to be discovered as the result of its relative smallness, imperceptible against the background of the high total content of this form of phosphorus.

Unlike all the other fractions, PhP takes up practically on P^{32} during the first 40 hours of development. Minute uptake of the isotope becomes apparent only at later stages and shows a gradual increase toward the end of development.

RNA. The RNA phosphorus content for sevriuga ova amounts to an average of 7.5 μg per ovum, i.e., about 17% of total phosphorus in the ovum; for beluga ova the figure is about 20 μg (about 22%). Equivalent values were obtained on determination of RNA in terms of ribose.

At the beginning of development the amount of RNA shows no noticeable change and increases somewhat toward the end of development (Fig. 7). However, in this fraction intensive phosphorus exchange is found: even in the unfertilized ovum we found about 20% of the total P^{32} of the ovum in the RNA; the isotope must have been taken up during the period of maturation of the ovum. After fertilization the relative and specific activities of RNA phosphorus increase fairly rapidly, but beginning with the 40th hour of incubation there is a tendency to a decline in these indices. This may be connected, as in the case of PhL, with lowering of the activity of the acid-soluble fraction from which the precursors for RNA synthesis are drawn.

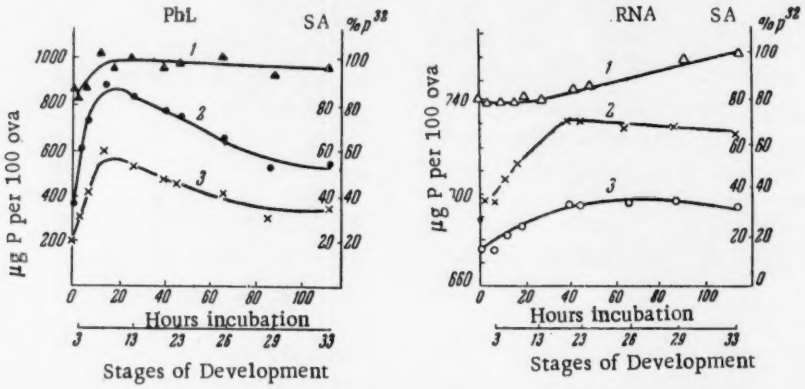

Fig. 7. Change in the content and relative and specific activities of phosphorus in the phospholipid and RNA fractions during development of sevriuga ova: 1) content P; 2) specific activity; 3) relative activity.

DNA. Beluga roe contains at the beginning of development 1.5 μg DNA per ovum. Later the amount of DNA gradually increases, reaching a value of 7-8 μg by the time of hatching. More detailed observations were made on sevriuga ova with fairly frequent sampling in the initial period of development.

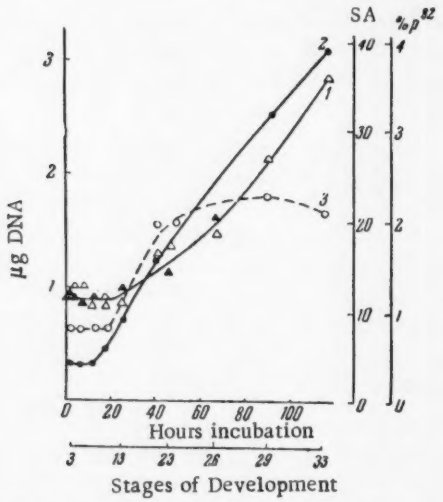

Fig. 8. Change in the content and relative and specific activities of DNA during development of sevriuga ova: 1) DNA content per 1 ovum; 2) relative activity; 3) specific activity.

About 0.9 μg DNA is found in the unfertilized sevriuga ovum (Fig. 8). The same amount of DNA is found in the mature ovum, ready for fertilization. Identical results were obtained when DNA was determined in terms of phosphorus and in terms of desoxyribose. After fertilization and throughout the whole period of division the DNA content of the ova remains practically unchanged and only beginning with stage 13, i.e., beginning of gastrulation does the amount of DNA begin to increase; this increase continues up to the end of the period studied.

The relative and specific activities of DNA phosphorus also show no increase up to stage 13, and only after the onset of gastrulation begin to increase sharply, as can be seen from Fig. 8. After 60 hours' incubation the SA of DNA, unlike its relative activity, begins to decrease. The reasons underlying this difference in behavior of the two indices will be considered in the discussion of results.

Residual phosphorus. The protein residue left after isolation of all the phosphorus-containing fractions, which includes practially all the proteins of the ovum, contains an extremely small amount of phosphorus which

could not be determined sufficiently accurately by chemical means. This fraction contained from 0.2 to 1.0% of total P^{32} content; this amount may be regarded as negligible if the huge mass of the protein residue is taken into account.

DNA Content in Sevriuga Spermatozoa

Amount of DNA in the spermatozoon, 10^{-6} μg		
In terms of phosphorus	In terms of desoxyribose	In terms of absorption at 265 mμ
2.31	2.08	2.50
2.44	2.09	2.48
2.55	2.20	2.52
Average: 2.43	2.12	2.50

DNA content of sevriuga spermatozoa. According to our calculations, 1 ml of sevriuga sperm contained $2.925 \cdot 10^6$ cells.

The results of DNA determination in sevriuga spermatozoa are summarized in the table, calculated per one cell.

When DNA content was determined in terms of phosphorus the amount of phosphorus found was multiplied by a factor of 10.3 (based on the assumption that the phosphorus content of sevriuga DNA was 9.6% as in the majority of animals). When the determination was made in terms of desoxyribose the DNA content was judged by comparing the intensity of the Dische color reaction [11] in the sample tested with a standard solution of DNA from calf thymus.

For determination of the DNA content by means of ultraviolet absorption a comparison was made between absorption at 265 mμ in the DNA fraction of the sperm and in the perchloric acid hydrolyzate of a standard solution of DNA from calf thymus.

The results obtained by the different methods showed fairly good agreement. It must, however, be borne in mind that the determination in terms of desoxyribose by the Dische method is more specific than the other two methods which may give somewhat high results.

DISCUSSION

If the analytical data obtained create the impression that the majority of the phosphorus compounds undergo only slight quantitative changes during the course of development, the use of radioactive phosphorus isotope demonstrates the presence of intense exchange between fractions containing phosphorus compounds from the very beginning of embryonic development.

Moreover, on the basis of the data obtained, it is even possible to postulate certain views concerning phosphorus metabolism at the final stages of maturation of the ova.

First of all, there is no doubt that during the period elapsing between the injection of gonadotropic hormone and ovulation, intense exchange of phosphorus occurs between the ovum and the maternal organism. This exchange is mediated by low-molecular phosphates of the acid-soluble fraction which act as the "common fund" which takes up P^{32} brought to the ovary by the blood stream and from which phosphorus is drawn for entry into the other fractions. The fate of P^{32} in other fractions at the final stages of maturation of the ova has not been traced by us, but judging by the values of their activity during the first hours of development some impression can be gained with respect to their exchange during this period. Thus, the RNA fraction before fertilization of mature ova already contains 18% P^{32}, which indicates the presence of fairly vigorous RNA exchange at the last stages of maturation of the ovum. The same may be said of the labile phosphate and PhL fractions. Detailed studies of phosphorus exchange during this period are of considerable interest for the understanding of the biochemical essence of the process of maturation of ova and for deciphering the mechanism of action of gonadotropic hormone, injections of which are used extensively in sturgeon breeding.

Examination of all the data together, as shown by curves of changes in radioactivity of phosphorus fractions during development reveals a striking tendency toward increasing activity at the beginning of development in all the acid-insoluble fractions (with the exception of PhP), replaced by a drop of activity toward the end of development. As already noted, this does not signify a decline of their exchange. All these fractions undergo exchange with the acid-soluble fraction, enriching themselves at its expense with the isotope so long as its concentration in the acid-soluble fraction is relatively high. Later the P^{32} concentration in this fraction decreases and an interrelation of activities is created which leads to exchange with this fraction, resulting in loss of isotope from the acid-insoluble fractions.

This exchange is also connected with continuous passage of phosphorus from the acid-soluble fraction to the acid-insoluble fractions, whose phosphorus content slowly increases (with the exception of phosphoproteins). Nevertheless, the P content of the acid-soluble fraction also increases in the course of development. Evidently, the loss of isotope from this fraction is associated not only with its "outflow" and exchange for nonradioactive phosphate of other fractions but also with the entry of new amounts of nonradioactive phosphorus into the acid-soluble fraction. The source of this phosphorus may be supposed to be the yolk PhP. These, apparently, constitute a reserve form of protein and phosphorus, which is supported by their predominant localization in the yolk as demonstrated by preliminary analyses of isolated yolk of the ova. The absence of appreciable decrease in PhP phosphorus during development is explained by the relatively small amounts of phosphorus given off by them. Such function of PhP explains the noninclusion by them of P^{32} during most of the period of embryonic development – they participate in the exchange unilaterally, contributing their phosphorus to the common fund as inorganic phosphate. That small P^{32} uptake by PhP which becomes noticeable at late stages of development must, in our view, reflect the synthesis of new PhP which, unlike the reserve ones, can be designated as functional PhP similar to those found in the brain [12] and other tissues.

On the basis of all the above, the course followed by phosphorus in developing ova of sturgeon may be described as follows. The acid-soluble fraction constitutes a common fund of phosphorus; it takes up P^{32}, principally in the form of inorganic phosphate. A considerable part of the latter enters into high-energy phosphates of the acid-soluble fraction as the result of oxidative phosphorylation. The P^{32} taken up by RNA and DNA and probably also PhL and functional PhP (whose exchange depends on high-energy bond phosphorus compounds [12-14]) during the course of development is drawn from the fraction of labile phosphate. The continuous "outflow" of mineral phosphate from the acid-soluble fraction is evidently compensated at the expense of dephosphorylation of yolk PhP.

The phospholipids together with the acid-soluble fraction are one of the most energetically exchanging fractions, the intense renewal of PhL phosphorus beginning during the first hours of development. This may be associated with high mitotic acitivity during this period. The work of Levin et al. [15] points out that there is in fact close connection between the intensity of P^{32} uptake by PhL and mitotic activity in regenerating liver.

The data obtained by us with respect to the content and renewal of phosphorus in DNA force us to touch on questions which are of theoretical interest for embryonic chemistry.

As already mentioned, the DNA content of sevriuga roe from the moment of fertilization to the beginning of gastrulation remains the same. There is no formation of DNA during this period as shown also by the fact that the relative and specific activity of DNA phosphorus do not increase during this time.

Appreciable synthesis of DNA only begins from the gastrulation stage and continues to the end of the period studied.

Analogous data were obtained from developing ova of R. platyrhina by Hoff-Jorgensen and Zeuthen [16]. On the basis of their findings, these authors concluded that there was no desoxyribose synthesis until late blastula stage. The synthesis of desoxyribose linkages (whose number was determined by the microbiological method) begins only before the onset of gastrulation. Later Lovtrup [17], also using the microbiological method, found that the DNA content of Amblystoma mexicanum ova remained constant during division; it began to increase at the late blastula stage.

Kutsky concludes from his data [18] that there is continuous increase in DNA content during development of R. pipiens, but she only carried out analyses at four stages. Sze [19], investigating the same animal, reached the same conclusion as Kutsky but found only slight increase in DNA content at the blastula stage; he found no increase in DNA later on (prior to onset of gastrulation). An appreciable increment in DNA in R. pipiens embryos can only be found after the beginning of gastrulation. This is exactly the conclusion arrived at by Gregg and Lovtrup [20] according to whom DNA synthesis in R. pipiens begins only on termination of division. In the latter investigation use was made of the microbiological method which is doubtless more specific than the usual chemical methods.

Comparison of literature data with our results shows definite similarity between sevriuga and amphibia with respect to the dynamics of DNA synthesis at early stages of development.

The question of DNA synthesis in developing ova is closely linked with the second important feature of DNA

exchange in them, which consists of the fact that extremely large quantities of this nucleic acid are present in the sturgeon ova at the beginning of their development.

If it is taken that each member of the mitotic tetrad (which, evidently, remains entirely within the limits of the egg, since the polar bodies formed during maturational divisions are extruded under the ovum membranes) contains as much DNA as the spermatozoon, then the theoretically expected amount of DNA in mature unfertilized sevriuga ovum should not exceed 10^{-5} μg. However, we found 0.9 μg DNA per ovum or approximately 100,000 times the theoretically expected amount. This apparently means that the unfertilized sevriuga ovum (also beluga ovum which contains even more DNA, viz.,1.5 μg) contains considerable amounts of "excess" DNA. It must be mentioned, it is true, that the value 0.9 μg may be somewhat high since it is obtained from chemical analyses which may give high results in the case of such material as amphibian and sturgeon ova. Thus, according to Lovtrop's data [17] DNA determinations by chemical means give results 8-10 times higher than those obtained by more specific microbiological methods. If this is taken into account, the value for "excess" DNA in sevriuga ova should, possibly, be reduced by one order. More precise determination of this value will require further investigation by more specific and sensitive methods.

The presence of such "excess" DNA in unfertilized ova has been demonstrated in a number of species.

Various authors found that the ova of the sea urchin contained amounts of DNA exceeding the amount of DNA present in one spermatozoon of this animal by 28-290 times [21-23]. An even greater difference is found in tailless amphibia: the ovum of R. platyrhina contains 5000 times the amount of DNA present in its spermatozoa [16], and in the ova of R. pipiens the amounts of DNA is 95,000 times that found in the nuclei of its liver cells [19]; the absolute amount of DNA in the unfertilized ovum of R. pipiens (0.96 μg) is very close to that found by us for sevriuga.

"Excess" DNA, as shown in R. platyrhina ova [16] and R. pipiens ova [19] must be localized outside the nucleus. These data are confirmed by direct analysis of nuclei isolated from R. pipiens ova prior to reduction-division [24]: it has been shown that the DNA content of such nuclei is approximately double the DNA content of this animal's spermatozoa.

There are references in the literature to the presence of extranuclear DNA in the ova of other animals as well. Thus, DNA was found in avidin [25] and yolk [26] of hen eggs.

Data on the DNA content of sevriuga and beluga ova and information regarding the volume of female pronuclei of these ova provide a basis for the conclusion, reached by calculations similar to those made by Sze [19], that all the DNA cannot be localized in the nucleus. Despite the considerable divergence in the values of "excess" DNA as determined by different authors, which is evidently connected with the imperfection of the methods used, we arrive at a common conclusion that "excess" DNA is contained in the unfertilized ova of a number of animals, including sevriuga and beluga; this DNA is most probably localized in the cytoplasm and serves as reserve material for the formation of the nuclear apparatus of the newly formed cells. The presence of such a reserve appears to be essential during the period of division when the formation of cells proceeds at the maximal rate and the synthesis of DNA de novo, as far as can be judged from the data available at present, does not take place.

Another important feature of DNA exchange becomes apparent on consideration of our data. Comparison of changes in relative and specific activity of DNA and RNA reveals that in the case of RNA both indices diminish toward the end of development whereas in the case of DNA only the specific activity drops, the relative activity continuing to increase.

This difference is associated with the well-known metabolic stability of DNA. New molecules of DNA are formed at each division of the cell but, once formed, they are no longer renewed [27]. The continued increase of relative activity of DNA despite the decrease of radioactivity of the acid-soluble fraction (from which the precursors for DNA synthesis are drawn) is explained by the irreversibility of P^{32} uptake by DNA. At the same time, with each successive mitosis fewer and fewer phosphorus atoms taken up by the DNA formed are radioactive; this results in the curve of DNA specific activity showing a maximum, with an increase followed by a drop to a level almost equal to the specific activity of labile phosphate.

The RNA fraction exhibits a tendency, in the second half of the incubation period, to diminution not only of the specific but also of the relative activity. This indicates that RNA phosphorus undergoes practically continuous exchange with the medium. Differences in RNA and DNA exchange thus become apparent and are expressed

in the fact that RNA takes up P^{32} reversibly while DNA does so practically irreversibly; these differences are evidently associated with differences in the function of these two types of nucleic acids.

The importance of RNA in embryonic development has been firmly established by a number of workers. The RNA content in embryos and their various parts increases with development, the intensive synthesis of RNA being associated with periods of vigorous growth and differentiation of tissues [28]. In sevriuga embryos we found a small increase in the amount of RNA beginning with stage 26 when the foundations of the main parts of the body had already been laid down and the differentiation of their tissues and synthesis of specific tissue proteins was beginning. It will be shown in the next communication that the increase in RNA content precedes the considerable increase observed in apyrase activity after stage 28 (this increase appears to be connected with the formation and growth of muscle tissue) and the sharp increase in alkaline phosphatase activity.

The relative constancy of RNA content at the early stages of development is probably explained by the fact that the ovum is rich in this substance from the very beginning of development. In this sense there is similarity between the behavior of RNA and DNA. The essential difference is constituted, however, by the intense renewal of RNA from the very start of development, against a background of its unchanging total content, whereas in the case of DNA the beginning of P^{32} uptake coincides with the beginning of increase in this amount.

In this connection it should be mentioned that according to Kutsky [18] the renewal of phosphorus in RNA increases noticeably with the beginning of gastrulation. Unlike the case of R. pipiens, which provided these data, in sevriuga, P^{32} is taken up energetically by RNA from the very beginning of development and no substantial change can be discerned in the rate of RNA phosphorus renewal at the onset of gastrulation.

Nevertheless, our other results confirm the widely held concept of the beginning of gastrulation, one of the most important stages in the morphogenesis of the embryo, being a critical moment with respect to certain aspects of metabolism as well [29]. We have already indicated that DNA synthesis in sevriuga embryos begins almost simultaneously with the onset of gastrulation. Evidently, a fundamental change in DNA metabolism is associated with gastrulation. Also connected with it is the change of energy exchange in sevriuga embryos. The increase observed between the 20th and 30th hour of incubation of the ova in the rate of labile phosphate renewal and loss of isotope from inorganic phosphate expressed in a rapid drop of relative activity apparently reflects the rising predominance of the importance of phosphorylation mechanisms at the stage of gastrulation.

SUMMARY

The content of phosphorus in the main phosphorus-containing fractions was determined at various stages of embryonic development of sevriuga (Acipenser stellatus) and of beluga (Huso huso). In the course of development the phosphorus content rises somewhat in the inorganic phosphate, phospholipid, RNA and DNA fractions, remains unchanged in the phosphoprotein fraction and decreases in the labile phosphate fraction.

P^{32}, administered to the female sevriuga intramuscularly in the form of $Na_2HP^{32}O_4$ simultaneously with gonadotropic hormone, penetrates rapidly into the ovarian ova, being taken up first of all by the acid-soluble fraction. Investigation of the distribution of P^{32} between the different fractions at various stages of embryonic development has established the presence of intensive phosphorus exchange in most of the fractions studied.

The DNA content of sevriuga spermatozoa has been determined. It is $2.12 \cdot 10^{-6} - 2.50 \cdot 10^{-6}$ μg per one cell. The large difference between this value and the DNA content of mature unfertilized sevriuga ova (0.9 μg DNA per ovum) suggests the presence of considerable amounts of "reserve" DNA in the latter.

The significance of the data obtained is discussed from the point of view of the association of metabolism of different phosphorus fractions with certain points in the embryonic development of sturgeon.

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QUANTITATIVE ASSAY OF AMINO ACIDS IN MILK PROTEINS

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Paper chromatography is used extensively in the studies of protein metabolism and in investigations of the structure of various proteins and their amino acid composition.

A large number of methods have been suggested for the quantitative estimation of amino acids after their partition by paper chromatography; we have checked and adopted the following of these methods: 1) ninhydrin method using special parchment paper in Sdobnikova's modification [1]; 2) ninhydrin method on chromatographic paper designated "rapid" by Paskhina, using Troll and Cannon reagents [2] and 3) Bode's method [3].

Method 1 in Sdobnikova's modification failed to give satisfactorily reproducible results since the color of eluates obtained on treatment with ninhydrin proved to be extremely unstable (it altered to the extent of 55-60% even during brief periods of photometric examination).

The method described by Paskhina [2] has the disadvantages of requiring the use of such reagents as potassium cyanide, freshly distilled phenol and pyridine; the use of these requires great care and good fume cupboard equipment. Moreover, the reaction of ninhydrin complex formation in the presence of cyanide is sensitive even to traces of ammonia which necessitates purification of all working solutions with permutite, a wasteful procedure. These disadvantages become considerable on transition to large-scale analyses.

Bode's [3] method proved to be the most satisfactory; it is used in our department for quantitative estimation of certain amino acids after their separation by chromatography.

The technique of applying this method to milk protein hydrolyzates is described in the present work.

Quantitative determinations of amino acids were carried out on proteins isolated from milk. These preparations can be stored in the dry state for considerable periods of time thus facilitating work on a large scale.

METHOD

Isolation of proteins from milk and their preparation for analysis. Average samples of milk were taken during consecutive 48 hours, proportionally to the milking yield. The proteins were isolated from skim milk after twice-thrice repeated separation. Precipitation of casein was effected by 5% solution of acetic acid. The casein precipitate was filtered off, washed with distilled water and reprecipitated twice. For this purpose casein was dissolved in a minimal volume of 0.1 N NaOH and reprecipitated from solution by 5% acetic acid, then washed thoroughly with water and subjected to another reprecipitation.

Water-soluble proteins (albumins, globulins) were isolated by heating the filtrate obtained after the first precipitation of casein to 90° and maintaining it at this temperature for 30 minutes. The precipitated protein (mixture of albumin and globulin) was treated similarly to the casein, i.e., filtered, washed with distilled water and reprecipitated. The isolated protein preparations – casein and mixture of albumin and globulin – were kept at room temperature and allowed to dry in air; they were then pulverized.

The protein preparations were characterized by determinations of moisture content, ash and nitrogen.

Hydrolysis. A weighed sample of protein was covered with a 20-fold volume of 6 N HCl and hydrolyzed in an autoclave for 4 hours at 3 atmospheres. The humins were filtered off and hydrochloric acid removed by re-

peated vacuum distillations. The solid residue was dissolved in distilled water. With a sample of 100 mg the final volume of hydrolyzate should be 2.5 ml.

Chromatographic separation. Chromatograms were obtained on "rapid" chromatographic paper made at the Volodarskii Paper Mill in Leningrad. Sheets of paper measuring 25 × 70 cm were first freed from metallic cations by moistening them with a 0.2% solution of 8-hydroxyquinoline in a mixture of n-butyl alcohol, glacial acetic acid and water taken in the ratio 4 : 1 : 1. The moist sheets were placed in chromatographic baths and washed with water-saturated n-butyl alcohol and acetic acid in the ratio indicated. The metallic complexes with hydroxyquinoline moved near the front of the solvent as a dark strip. Washing was continued until the solvent flowed out; this usually took no less than 48 hours. The paper was then dried at room temperature, after which it was entirely suitable for quantitative determination of amino acids (as recommended by T. S. Paskhina).

Application of the solutions under investigation to the paper was carried out with the help of precalibrated micropipets. It has been established that the best results are obtained on application of not more than a 0.003 ml solution. A larger diameter of the initial spots on chromatograms makes them diffuse and blurred after development. When large amounts of solution have to be applied, repeat applications must be resorted to. In order to obtain a visible spot on the chromatogram (using ninhydrin solution as developer) it is sufficient to have not less than 0.05 μmole of amino acid in the spot.

It follows from this that the amounts of milk protein hydrolyzate required for separation and determination of the amino acids contained in it should be such as to correspond to not less than 100 μg amino acid.

Some amino acids can be separated successfully by means of 1-dimensional chromatograms with repeated passage of one solvent.

The already mentioned mixture was used as solvent: n-butyl alcohol, glacial acetic acid, water (4 : 1 : 1). Repeated passage of this mixture seven times through the chromatogram with casein hydrolyzate applied to it resulted in the separation of the following amino acids: cystine, lysine, histidine, arginine, aspartic acid, serine, glycine, glutamic acid, threonine, alanine.

The time and rate of movement of solvent passed 7-times (20°) were as follows:

Sequence of passage	Distance covered by solvent, in cm	Time of passage in in hours
1	22	5
2	25	6
3	40	12
4	66	17
5	70	19
6	Solvent flowing out	20
7	Solvent flowing out	24

After the first passage of solvent, the chromatogram was dried in air, replaced in the bath fitted in the chamber and subjected to another passage of solvent, and so on.

Such amino acids as proline, tyrosine, methionine, valine, phenylalanine, leucine and isoleucine are lost when the solvent flows out. Separation of these amino acids may be achieved on a parallel chromatogram using a different procedure of solvent passage: 1) for 30 cm, 2) for 50 cm and 3) to the edge of the sheet, (not allowing the solvent to flow out), or by the method recommended by Zaitseva and Belozerskii [4].

Quantitative estimation of amino acids. The principle of the Bode method [3] consists of the following: the ninhydrin-developed chromatogram is treated with a solution of $Cu(NO_3)_2$; the violet color of the complex obtained by the reaction of amino acids with ninhydrin changes to ruby red as the result of the formation of copper derivatives. The colored complex is extracted with methyl alcohol. The optical density of the solution obtained is measured photoelectrocolorimetrically; the amino acid content is determined from calibration curves. The method's inventor gives two curves: one for tyrosine and phenylalanine, the other for serine, glycine, valine, alanine, norvaline, leucine, methionine, arginine, histidine, aspartic acid and glutamic acid. However, there are data indicating that each amino acid should have its own calibration curve, since the intensity of color is not equivalent to the molarity of the solutions [5].

Calibration curves are plotted from the optical density of standard solutions of amino acids prepared with twice-distilled solvent. The amino acid preparations are first dried to constant weight. Amino acid solutions are applied to the paper in amounts of from 2.5 to 12.5 μg; the chromatographic analysis is then carried out step by step under conditions identical with those for chromatographic analysis of protein hydrolyzates.

ANALYTICAL PROCEDURE

Following chromatographic separation of the hydrolyzate (as described above) the chromatogram was dried in air at room temperature until no more odor of solvent could be detected, moistened with ninhydrin solution (90 ml 0.5% solution ninhydrin in acetone + 5 ml glacial acetic acid + 5 ml water) and the color developed by warming in the drying cupboard for 15 minutes at 30°; colored spots of ninhydrin complex with amino acids appeared on the chromatogram. The chromatogram was then moistened with copper solution (1 ml saturated aqueous solution of $Cu(NO_3)_2$ mixed with 0.02 ml 65% HNO_3, made up to 100 ml with acetone and kept in the dark for 30 minutes). The amino acid spots became ruby red as the result of this treatment; their contours were traced in pencil and cut out. Blank samples of equal area were cut out from the free background of the chromatogram. It is desirable to cut out such areas at the same level as that occupied by the amino acid being determined. The excised areas with amino acids and blanks were cut up with scissors, placed in test tubes and covered with 10 ml methyl alcohol.*

Extraction time is determined by the completeness of elution of the colored substance from the paper. It has been observed that the amino acids lysine, histidine, arginine, aspartic acid (situated nearer the starting line) give more blurred and diffuse spots when the described chromatographic method is used, and their extraction is complete before that of serine, glutamic acid, threonine and alanine. However, extraction of all the amino acids is complete at the end of 2 hours. Repeat colorimetry of the same solutions after 5, 15 and 24 hours shows no change and indicates completeness of extraction and stability of the colored solutions.

Determination of glutamic acid, whose content in casein amounts to over 20%, presents certain special features. Under our analytical conditions the amount of this acid in the chromatogram spot is about 20 μg. It is known [5] that optimal concentrations of amino acids, with preservation of a linear relationship between concentration and color, lie within the limits 2.5-12.5 μg. Extraction of glutamic acid was therefore effected with 20 ml methyl alcohol. The concentration of the resultant solution was then within the limits indicated.

The optical density of the solutions was measured against blanks in a photoelectrocolorimeter "FEK-M" with filter No. 3 and 20 mm cuvettes. The amounts of amino acids are found from corresponding calibration curves prepared as described above.

VERIFICATION OF METHOD

The results of photometric examination of standard solutions of some amino acids are given in Table 1.

TABLE 1

Photometric Data on Standard Solutions of Amino Acids

Extinction values

Amino-acid	Amino acid content in solution in μg			
	5	8	10	12,5
Lysine	0.060	0.108	0.140	0.180
Histidine	0.013	0.025	0.033	0.044
Arginine	0.055	0.084	0.105	0.129
Aspartic acid	0.045	0.076	0.096	0.122
Serine	0.072	0.118	0.148	0.186
Glycine	0.140	0.200	0.242	0.292
Glutamic acid	0.090	0.150	0.190	0.240
Threonine	0.080	0.140	0.179	0.228
Alanine	0.100	0.159	0.198	0.248

* Bode [3] used 8 ml methyl alcohol for the extraction.

Table 2 presents data on determination of the content of certain amino acids in casein. Literature data are given for comparison.

TABLE 2

Amino Acid Content of Casein (in %)

Amino-acid	Literature data			Own data
	Block, Bolling [6]	Tristram [7]	Ermakova [8]	
Lysine	6.9	8.2	8.7	5.8
Histidine	2.5	3.1	3.6	2.7
Arginine	4.1	4.1	4.0	3.7
Aspartic acid	6.3	7.1	7.0	6.1
Serine	6.5	6.3	6.7	5.6
Glycine	0.5	2.0	2.6	2.0
Glutamic acid	22.8	22.4	20.1	22.2
Threonine	3.9	4.9	5.9	4.0
Alanine	5.6	3.2	3.8	3.5

Table 2 shows that our own data agree with data cited in the literature.

SUMMARY

Three methods of quantitative estimation of amino acids after their separation by paper chromatography were checked and the most suitable one for large-scale work was found to be the method of Bode which can be recommended for quantitative assay of amino acids in milk proteins.

Optimal conditions for good separation of 10 amino acids on a 1-dimensional chromatogram with fractional passage of solvent have been found.

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RIBOFLAVIN-SENSITIZED PHOTO-OXIDATION OF PHENOLS

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Riboflavin, in the form of nucleotides, is an active component of a number of oxidation-reduction enzymes. It also has photosensitizing properties and can participate in oxidation-reduction reactions initiated by light. Owing to these properties riboflavin, like chlorophyll , evidently occupies a definite place in the interrelations of the organism and light, especially in respiratory metabolism of plants.

In our investigations of the chemistry of green plant respiration in the dark and in the light and the interrelations of respiration and photosynthesis, we studied the role of flavoprotein oxidases and polyphenoloxidases in the above-mentioned phenomena [1-6]. The present work deals with our investigations of riboflavin-sensitized photo-oxidation of phenols commonly used in studies on polyphenoloxidase. Some other substances were also used in these studies on the chemistry of photo-oxidation.

METHODS

Photo-oxidation of phenols was studied in a luminescent Warburg apparatus adapted for this purpose. The basic principle of this apparatus was taken from Schenck [7].* Six luminescent lamps "B-S-30", three lamps along each long wall of the apparatus, were fitted inside the Warburg apparatus bath. Two lamps were placed parallelly side by side along the horizontal and the third lamp above these (underneath the slit between them). During operation of the apparatus the lamps were in water. The lamps were mounted so that they could be replaced at any time. The vessels for the substances being investigated (8 on each side) were placed in the bath in such a way that their bottoms were directly above the two upper lamps, 2 mm above the latter; the diameter of the bottom of these vessels was 4 cm. Such placement of the vessels enabled the same amount of light to be supplied to each one of them. The manometers were rocked horizontally.

Experiments were carried out at 25°; this temperature was maintained at a constant level by means of an ultrathermostat. Into each vessel was placed 3.5 ml distilled water or phosphate buffer solution, pH 5.6 and 1.0 ml aqueous solution of the substance under investigation. The total volume of the mixture was 5 ml. To the central extension of the vessel was added 0.3 ml 20% KOH or water, depending on the aim of the experiment. After incubation, hydrogen peroxide was determined in the samples iodometrically or manometrically. When the former method was used 1 ml 1 % solution KI and 0.5 ml 20% H_2SO_4 were added to the samples; these were allowed to stand at room temperature in the dark for 15 minutes after which the liberated iodine was titrated with 0.01 N hyposulfite in the presence of 1-2 drops of 10% starch solution. When the manometric method was used, incubation was followed by addition of 0.5 ml concentrated solution of crystalline catalase** to the side neck of the vessel; determination of liberated oxygen was carried out in the usual way in the Warburg apparatus. It was found that 10 minutes was sufficient for the catalase to decompose all the accumulated peroxide and for gaseous equilibrium to be established in the manometers.

The amounts of peroxide in the tables are everywhere expressed in μliters of oxygen liberated by the catalase. In the case of the iodometric method, conversions from milliliters of 0.01 N hyposulfite to oxygen were made on

* The apparatus was constructed from our diagram by A. E. Popov, Mechanic at the A. N. Bakh Institute of Biochemistry. AN SSSR.

** Catalase was obtained from bovine liver by the Sumner and Dows [8] method by E. I. Petrochenko, Laboratory Assistant.

the basis of titrated samples. This was achieved by determination of hydrogen peroxide in identical samples of the latter by the iodometric and the manometric methods,and the results obtained were used for calculating the conversions.

RESULTS

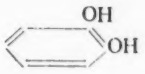
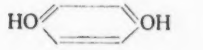
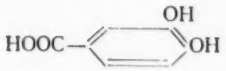
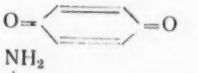
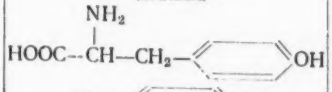
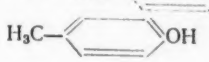
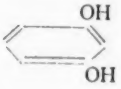
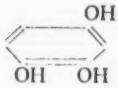
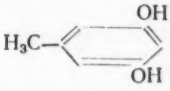
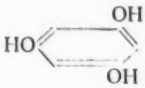
The experimental results are given in Tables 1 and 2.

Without riboflavin all the substances investigated did not absorb oxygen under these experimental conditions, or only absorbed a few μliters as shown for pyrocatechol (Table 1).

TABLE 1

Riboflavin-sensitized Photo-Oxidation of Phenols

Duration of incubation 1 hour; 25°, pH 5.6

Substance	Formula	O_2 absorbed in liters	Amount of peroxide in liters O_2 *
Control (aqueous solution of riboflavin)		4.5; 4.6	1.2
Pyrocatechol		16.8; 18.4	7.2
The same, without riboflavin		8.5; 9.4	6.8
Hydroquinone		16.5; 13.4	8.4
Protocatechuic acid		13.8; 15.6	—
Gallic acid		14.7; 13.8	—
p-Benzoquinone		0	—
Tyrosine		70.5; 80.8	25.2
p-Cresol		80.5; 86.9	24.0
Resorcinol		55.2; 49.3	18.6
Pyrogallol		117.6; 117.3	55.2
Orcinol		126.0; 135.4	13.8
Phloroglucinol		243.8; 268.8	39.8
Tannin from tea	—	101.5; 85.0	42.0

* Peroxide determined iodometrically.

It can be seen from Table 1 that aqueous solution of riboflavin (control), exposed to light, elicits negligible absorption of oxygen and formation of peroxide (it must be noted that on increasing the amount of riboflavin two-fold the absorption of oxygen and accumulation of peroxide shows a three-fold increase).

Oxygen absorption by pyrocatechol, hydroquinone, protocatechuic acid and gallic acid in the presence of riboflavin is somewhat greater than the control but is still very small. On the other hand, tyrosine, p-cresol, resorcinol, pyrogallol, orcinol, pholoroglucinol and tea tannin show considerable increase in oxygen absorption and peroxide accumulation in the presence of riboflavin. Consideration of the structural formulas of these substances reveals that there is a relationship between photo-oxidation and structure. Para- and ortho-diphenols and their derivatives show little tendency to undergo oxidation. Derivatives of monophenol such as p-cresol and tyrosine are readily oxidized; this is even more true in the case of metadiphenol — resorcinol, as well as orcinol, pyrogallol, phloroglucinol which can be considered as derivatives of metadiphenol.

The amounts of peroxide given in Table 1 were determined iodometrically. This method, however, also estimates quinones which are usually found among the oxidation products of phenols. Table 2 gives data obtained from peroxide estimations by the manometric method using crystalline catalase. The values are the average of two parallel estimations. Parallel convergence is indicated in Table 1.

TABLE 2

Formation of Hydrogen Peroxide on Photo-Oxidation of Phenols and Other Substances in the Presence of Riboflavin

(Duration of incubation 1 hour; pH 5.6)

Substance	O_2 absorbed in μliter	CO_2 liberated in μliter	Amount of H_2O_2 in μliter O_2	
			With catalase	Iodometrically
p-Cresol	81	26	23.4	21.6
Pyrogallol	117.4	55.6	55.2	55.2
Phloroglucinol	256.3	102.1	38.0	26.4
Pyrocatechol	10.8	—	0	8.4
The same + ascorbic acid	380.2	—	94.5	80.4
Ascorbic acid	375.4	—	91.8	84.0
The same without riboflavin	18.8	—	0	2.4
Hydroquinone	8.0	—	0	2.5
Dihydroxymaleic acid	157.7	—	39.9	—
The same without riboflavin	67.7	—	0	—
p-Benzoquinone	0	0	0	—

Catalase is known to decompose, with evolution of molecular oxygen, only hydrogen peroxide and its mono-substituted derivatives such as monoethylhydroperoxide. It does not decompose quinones. Table 2 shows that the amounts of peroxide measured manometrically and iodometrically are very similar. This indicates that hydrogen peroxide and not quinones is formed in the process of sensitized photo-oxidation.

DISCUSSION

Oxidation of phenols in the presence of riboflavin and on exposure to light has been described in the literature.

Andreae [9] in his model experiments observed oxidation of manganese and some phenol derivatives, e.g., p-cresol, in the presence of riboflavin and on exposure to light. Catalase inhibited oxidation of manganese. The author considers that in this case the reaction follows the course depicted below:

$$\text{Riboflavin} \xrightarrow{\text{light}} \text{activated riboflavin (RF*)}$$

$$\text{RF*} + \text{hydrogen donor (D)} \longrightarrow \text{reduced riboflavin } (RFH_2) + \text{oxidation product of D}$$

Oxidation product of D + reduced manganese (Mn^{++}) $\rightarrow$ D + oxidized manganese (Mn^{+++})

$RFH_2 + O_2 \longrightarrow$ riboflavin + H_2O_2

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2} O_2$$

D could be, for example, p-cresol.

Recently, Vernon and Ihnen [10] found that photo-oxidation of 2, 6-dichlorophenolindophenol + ascorbic acid by sugar beet homogenate was stimulated by riboflavin-5-phosphate. The authors consider that the reaction in the homogenate proceeds as follows:

$$\text{Chlorophyll} \xrightarrow{\text{light}} \text{activated chlorophyll (Chl}^*\text{)} + \xrightarrow{+O_2} \text{Chl}^* O_2$$

$Chl^* O_2$ + 2, 6-dichlorophenolindophenol, reduced $\longrightarrow$ chlorophyll + 2, 6-dichlorophenolindophenol, oxidized + H_2O_2

2, 6-dichlorophenolindophenol, oxidized + ascorbic acid $\longrightarrow$ 2, 6-dichlorophenolindophenol, reduced + dehydroascorbic acid

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2} O_2$$

In the presence of light riboflavin-5-phosphate acts similarly to chlorophyll.

In the works cited indications of hydrogen peroxide formation are either indirect or presumptive. Our data, on the other hand, demonstrate that hydrogen peroxide is in fact produced during riboflavin-sensitized photo-oxidation of phenols and certain other substances.

It follows from the reports cited [5, 6] that there are different hypotheses with regard to the mechanism of sensitized photo-oxidation. Some suggest initial activation of the sensitizing agent which then activates the substrate and the latter becomes able to combine with molecular oxygen. Others suggest that activated sensitizing agent combines with and activates molecular oxygen which becomes capable of oxidizing substrates (see also [11, 12]).

As already mentioned above, riboflavin enters into the composition of flavoprotein oxidases. When substrates are oxidized by these oxidases hydrogen peroxide is formed in accordance with the following scheme: protein-riboflavin + H_2 substrate $\rightarrow$ protein-riboflavin H_2 + substrate, oxidized; protein-riboflavin $H_2 + O_2 \longrightarrow H_2O_2$ + protein-riboflavin. That is, enzymatic oxidation of substrates is accompanied by transfer of hydrogen by these enzymes from the substrate to molecular oxygen with formation of hydrogen peroxide.

The material presented shows that the resultant effect of photo-oxidation with participation of riboflavin is analogous to enzymatic oxidation but, in the latter case, riboflavin is activated by proteins and in the former case it is activated by light. However, flavoprotein oxidases oxidize, in the dark, such substances as aldehydes, purines, amino acids, amines, glucose, glycolic acid etc., but not phenols. Thus it is, in this particular respect, that photo-oxidation differs from enzymatic oxidation. It is possible that activated riboflavin or light directly activate phenols which become capable of giving off their hydrogen to riboflavin. Further investigations are required before this can be clarified.

Tables 1 and 2 show that not all phenols can undergo sensitized photo-oxidation. Thus phenols such as pyrocatechol, hydroquinone, protocatechuic acid and gallic acid are practically not oxidized. Andreae [9] also showed that pyrocatechol and hydroquinone, unlike p-cresol, could not participate in oxidation of manganese in the presence of light and riboflavin.

These phenols are known to be oxidized to the corresponding quinones by enzymatic or catalytic (heavy metals) oxidation. For example, hydroquinone is oxidized to p-benzoquinone. Table 1 and 2 show that p-benzoquinone stops completely the absorption of oxygen in the presence of light and riboflavin. This could be regarded as confirmation of an inhibitory effect of quinones on riboflavin-sensitized photo-oxidation. Quinones are known to be readily reduced by ascorbic acid. This property of ascorbic acid was used by us in our experiments. Ascorbic acid did not, however, increase oxygen absorption in the presence of pyrocatechol (see Table 2) and the latter exerted no effect on riboflavin-sensitized photo-oxidation of ascorbic acid.

Consequently the failure of pyrocatechol oxidation is not caused by quinones. In this connection it should also be noted that oxidation of the other phenols investigated occurred through quinones but they underwent oxida-

tion on exposure to light very readily. The possibility is not excluded that photo-oxidation depends on the nature of the quinones formed, or may depend on the structure of the phenol being oxidized.

It is interesting to note that we had established earlier, [1] that there was increased oxygen absorption when the green centrifugate obtained from macerated barley leaves was exposed to light and decrease of oxygen absorption by similar preparations of kok-saghyz leaves [2]. It was found that no polyphenoloxidase could be detected by the usual methods in barley leaves and that the latter contained no substrate capable of being oxidized by polyphenoloxidase [5], whereas kok-saghyz leaves had both the one and the other [4]. Preparations of vine-leaves behave similarly to kok-saghyz leaves and also contain polyphenoloxidase and its substrates.

As already pointed out above, light probably activates riboflavin in a way similar to that of enzyme proteins, making it capable of removing hydrogen from substrates and transferring it to molecular oxygen, reducing the latter to hydrogen peroxide. If this is the case then the degree of oxidation of the substrate can be judged from the amount of oxygen absorbed and the amount of peroxide formed. Tables 1 and 2 show that in the control with riboflavin alone and also in experiments with p-cresol, tyrosine, resorcinol, ascorbic acid and dihydroxymaleic acid, catalase sets free one-fourth of the measured absorbed oxygen; with pyrogallol the value is one-half and with phloroglucinol it is one-fifth.

If it is assumed that on decomposition of H_2O_2 by catalase one half of the oxygen is liberated and the other half enters into the composition of water, it follows that upon oxidation of p-cresol, tyrosine, resorcinol, ascorbic and dihydroxymaleic acids half the absorbed oxygen enters the synthesis of hydrogen peroxide and the other half enters into the composition of the substance being oxidized, or possibly is also used in the biosynthesis of water.

With pyrogallol all the oxygen absorbed enters the biosynthesis of hydrogen peroxide; with phloroglucinol $^3/_5$ of absorbed oxygen is spent on formation of other oxidation products. These data indicate the different degrees of oxidation of these substances. What these differences consist of will be established on investigation of the products of oxidation.

SUMMARY

The active group of flavoprotein oxidases – riboflavin – can oxidize some polyphenoloxidase substrates (tyrosine, p-cresol, pyrogallol) on exposure to light, as well as ascorbinoxidase substrate – ascorbic acid and dihydroxymaleic acid oxidase substrate – dihydroxymaleic acid. In the presence of light, riboflavin also oxidizes the polyphenol widely encountered in plants – phloroglucinol, which is usually not oxidized by polyphenoloxidase or only oxidized very slowly.

The above-mentioned substrates are not oxidized in the dark by either riboflavin or flavoprotein oxidases. When these substances are subjected to riboflavin-sensitized photo-oxidation hydrogen peroxide is formed.

The facts obtained serve as further confirmation of the hypothesis postulated by us that light participates in plant respiration as a source of energy by way of the components of the respiratory mechanism.

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ENZYMATIC LINKS WHICH DETERMINE THE MAXIMAL RATE OF GLYCOLYSIS IN MUSCLE

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Modern data on the chemistry and kinetics of intermediate stages of glycolysis permit us to turn from measurement of the rate of glycolysis in the cell to determination of the factors on which one or another rate depends. The method by which glycolysis is inhibited during respiration (Pasteur effect) has been the subject of numerous and searching investigations. But the question of how the rate of glycolysis in the cell is fixed, and what determines its rise to various levels up to maximal glycolysis, has so far been barely touched. The slight increase in the rate of glycolysis which can be achieved in the resting cell by removal of the Pasteur inhibition proves to be quite insignificant when it is compared to the enormous rate of glycolysis during intensive work [1]. Therefore, investigation of the factors involved in the rate of glycolysis is a special problem which demands new methods for its solution.

The initial position usually assumed [2-7] is that the rate of glycolysis, as of any chain of enzymatic reactions, is determined by the rate of the slowest reaction in the chain. This should not however, lead to the view that in all states of the cell it is always the same factor that determines the rate of glycolysis. It is known that the rate of glycolysis varies over a wide range, e.g., in muscle 1000-fold [1, 8] from the minimal value at rest or during moderate work, when respiration serves as practically the only source of energy, to the maximal rise during strenuous work when only the coexistence of glycolysis and respiration and their intensive functioning can fulfill the vastly increased demands for energy [8, 9]. It would appear that in some conditions of the cell, at a certain range of the rate of glycolysis, limitation of the rate may be created by insufficiently rapid diffusion of the reacting substances; in other conditions this may be achieved by such factors as the limit of coenzyme concentration, action of inhibitors, limit of enzyme strength etc.

In short, the study of the glycolysis rate factor should, in our view, proceed according to the following scheme: functional state of the cell – range of the rate of glycolysis – narrow enzymatic link.

We studied the maximal rate of glycolysis, which corresponds to the extreme of strenuous cellular work. With this state of the cell the rate of glycolysis must evidently be determined by the strength of the enzymes only. The fact that during strenuous work there is no rate-limiting deficiency of coenzymes is supported by the remarkable stability of ATP level in muscle during tetanic contractions [10, 1] and in the brain during convulsive activity [11, 12]. But, if the limit of the rate of glycolysis depends on enzyme strength, then, according to the rule mentioned above, the factor determining the maximal rate must be the strength of the slowest enzyme involved in glycolysis. This implies not the number of turnovers but literally "molar strength", i.e., the product of the number of turnovers and enzyme content in the tissue [13, 14]. Maximal rate of glycolysis can be reproduced in tissue extract where, on the one hand, all inhibiting influences are removed, and on the other, optimal conditions are created for the action of enzymes. It is in such an extract that a search must be made for the slowest enzyme involved in glycolysis.

In our previous investigations [4-6] of the kinetics of the action of muscle HK and PFK* it was found that

* Designations: HK – hexokinase, PFK – 6-phosphofructokinase, TPD – triosephosphate dehydrogenase, LD – lactic-dehydrogenase, ATP and ADP – adenosine tri- and adenosine diphosphate, DPN – diphosphopyridine nucleotide, F-6-P – fructose-6-phosphate, F-1 : 6-P – fructose-1 , 6-diphosphate.

the power of these enzymes, expressed in values of Q, was considerably lower than Q for other individual enzymes involved in glycolysis and calculated from literature data [5]. This formed the basis for a hypothesis that the maximal rate of glucose glycolysis was determined by HK power [6] but that when glycogen formed the initial substrate and HK took no part in the process, the role of the limiting factor was taken by PFK [4, 5]. Naturally, so long as the rate of a complex many-stage enzymatic process was being considered, the data obtained from isolated enzymes could only serve as an indirect indication in favor of the suggested hypothesis rather than as proof thereof. Further investigation of this question on the complete glycolytic enzyme system was required.

The present work was based on the postulate that only that enzyme which accelerated the process when it was added or activated within the framework of the complete enzymatic system could be taken as limiting the rate of glycolysis. Such a criterion under optimal conditions for glycolysis permits direct identification of the enzyme which limits the maximal rate of glycolysis in tissue extract.

The rate of glycolysis was studied on a reconstructed system of dialyzed muscle extract and all the necessary additives, taken in optimal concentrations. When a linear relation was achieved between the rate of formation of lactic acid and concentration of muscle extract, one or another purified enzyme was added to the system and the rate of glycolysis determined anew.

The results of these studies confirmed our original suggestion regarding the influence of PFK and HK on the maximal rate of glycolysis.

PREPARATIONS AND METHODS

Muscle extract. Aqueous extract of rabbit skeletal muscles (according to Meyerhof) was neutralized and dialyzed against 0.15 M KCl. All operations were carried out in the cold room at 2-5°. Protein content was about 25 mg per 1 ml extract.

PFK. Highly purified preparation from rabbit muscle prepared by the present authors' method [4] and the method of Ling, Byrne and Lardy [15].

HK. Protein fraction III from baker's yeast, Cori's method [16].

TPD. Crystalline protein from rabbit muscle obtained by the Soreni method [17].

LD. Protein fraction from bovine heart, Straub method [18]. Isolation completed by second precipitation with $(NH_4)_2SO_4$.

Aldolase. Highly purified preparation from rabbit muscle according to Herbert, Green, et al. [19].

ATP. Obtained as the barium salt from alcohol-treated rabbit muscle by the usual method of mercury-barium reprecipitation. Purity of preparation in terms of myosin and PFK tests – 80% with respect to $\frac{1}{2}$ P_7 determined by acid hydrolysis.

ADP. Obtained by the myosin reaction from ATP by Liubimova's and Pevzner's method [20]. Purity with respect to P_7 – 90%.

DPN. Oxidized form of cozymase obtained from baker's yeast by the Le Page method [21] with Bresler's additions. Cozymase adsorbed on charcoal, eluted with pyridine and precipitated with acetone. Purity of preparation in terms of spectrophotometric test with TPD – 30%.

Glycogen. Obtained from rabbit liver in the usual way [22] and purified by repeated alcohol precipitation. Purity in terms of reducing properties of acid hydrolyzate – 90%.

F-6-P. Obtained as the barium salt by Neuberg's method [23]. Purity of preparation in terms of P and fructose – 90%.

F-1, 6-P. Acid barium salt obtained by the Neuberg method [23]. Purity of preparation in terms of P – 90%.

Lactic acid determinations were made by the colorimetric method of Barker and Summerson [24] with the use of p-hydroxydiphenyl.

EXPERIMENTAL

Maximal Rate of Glycolysis in Reconstructed Enzyme System of Muscle

The commonly used tissue preparations of muscle (macerated mass, homogenate) do not contain sufficient quantities of all the components needed for maximal glycolysis. Much better results with respect to an approach toward the maximal rate can be achieved by using a reconstructed system made up of dialyzed muscle extract and appropriate additives.

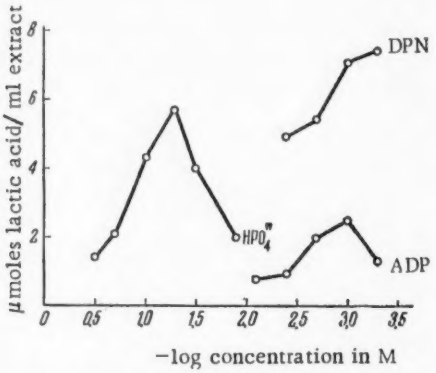

Fig. 1. Relation of rate of glycolysis to concentration of DPN, ADP and HPO_4.

We investigated the relation of the rate of glycolysis to the concentration of added coenzymes and other enhancing substances (Fig. 1).

A choice of optimal concentrations of all the components was made and the following glycolytic system was devised (final concentrations in moles per liter): ATP (ADP—$1 \cdot 10^{-3}$, DPN—$2.5 \cdot 10^{-4}$, $MgSO_4$—$3 \cdot 10^{-3}$, nicotinamide—$5 \cdot 10^{-3}$, F-1, 6-P—$1 \cdot 10^{-4}$, cysteine—$2 \cdot 10^{-3}$, carbohydrate (glycogen)—0.3% or glucose—$1.1 \cdot 10^{-2}$ or F-6-P—$1.1 \cdot 10^{-2}$), muscle extract—0.5 ml and phosphate buffer, pH 7.2—$8 \cdot 10^{-2}$ to a final volume of 2.5 ml.

By means of serial dilutions of the muscle extract such ratios of concentrations of the extract and the additives were found at which there was a linear relationship between the amount of lactic acid formed and concentation of the extract. The use of undiluted extract does not permit full demonstration of the extract's activity; only the rate of glycolysis measured on the linear portion of the kinetic curve enables correct assessment to be made of the glycolytic reserves of muscle extract. With this in mind, the initial rate, i.e., the number of micromoles of lactic acid formed during the first 15 minutes of incubation, was taken into account. The maximal rate of glycolysis in muscle extract was calculated on the basis of these data. If the rate of glycolysis is expressed in micromoles of lactic acid formed in 1 g of tissue in the course of one hour at 37° ("hourly rate") it can be seen that for the calf muscle of rabbit in situ this value is 1570 during tetanic contractions [10], for macerated skeletal muscle of rabbit it is only 70 [25] and for our reconstructed system prepared from muscle tissue the "hourly rate" of glycogen breakdown fluctuates within the range 1300-2680.

It was thus seen that it was possible to reproduce the true value of the maximum rate of glycolysis which develops in muscle during strenuous work (tetanus) by using muscle extract fortified with the necessary coenzymes, stabilizers etc. This conclusion enabled us to go on to experiments aimed at identification of the enzyme limiting the maximal rate of muscle glycolysis.

Effect of FPK on Rate of Glycolysis

If the linear course of glycolysis is established, not in relation to the substrate or coenzyme, but in relation to the concentration of muscle extract, it indicates that one of the enzymes contained in the extract exerts a limiting effect on the rate of glycolysis. If it proves possible to increase the rate of glycolysis in such an extract by adding a definite purified enzyme, it indicates that this is the particular enzyme that limits the rate of the reaction. We supposed, on the strength of our previous studies [5], that the role of this enzyme would be played by PFK.

Experiments revealed that addition of a relatively high concentration of PFK to undiluted muscle extract produced no change in the rate of glycolysis. However, as linear course of glycolysis was approached by serial dilutions of the extract, it proved possible to increase the rate of glycogen breakdown by addition of PFK (Table 1).

In further experiments the rate of glycogen breakdown was compared with the rate of glucose and F-6-P breakdown. It was found that the rates of glycogen breakdown and F-6-P glycolysis were kinetically different from that of glucose. It was also noted that in order to reach an approximately similar level of glycolysis, glucose breakdown required several tens of times higher concentrations of muscle extract than was the case for glycogen

and F-6-P. When the same concentration of PFK was added to all the samples, it was found that it produced no change in the rate of glucose breakdown, but increased the rate of glycogen and F-6-P breakdown 6-8-fold (Table 2). Such a marked increase in the rate of reaction was explained by the fact that the amount of PFK added was about 15 times higher than the intrinsic PFK content of these samples.

TABLE 1

Effect of Addition of PFK on the Rate of Glycolysis When the Latter Follows a Linear Course

(Composition of the reaction mixture as usual. Substrate – glycogen. PFK added to all samples ~50 μg protein. Incubation 60 minutes at 37°)

Concentration of muscle extract		Lactic acid formation in μmoles per 1 ml extract	
		Muscle extract (without addition)	Muscle extract with addition of PFK
Without dilution	1 : 1	102.6	101.2
Dilution	1 : 1	70.9	70.9
"	1 : 20	25.8	32.5
"	1 : 60	6.8	29.8
"	1 : 120	3.4	22.8

Acceleration of the process by addition of PFK was thus observed only when the course of glycolysis depended on the concentration of the extract and when glycogen or F-6-P and not glucose was being broken down.

TABLE 2

Effect of PFK on Rate of Glycolysis

(Composition of reaction mixture as usual. PFK content in samples 2 and 3 of muscle extract ~20 μg protein. PFK added to all samples 300 μg protein. Incubation 60 minutes at 37°)

Sample	Initial substrate	Concentration of muscle extract		Lactic acid formation in μmoles per 1 ml extract	
				Muscle extract (without addition)	Muscle extract with addition of PFK
1	Glucose	Without dilution		1.9	1.5
2	Glycogen	Dilution	1 : 40	0.93	11.9
3	F-6-P	"	1 : 40	2.6	16.3

We then traced the dynamics of the influence of various concentrations of PFK on the rate of the process. It follows from the Michaelis-Menten equation that with excessive concentration of substrate, when the enzyme is saturated with the substrate, forming an enzyme-substrate complex, the rate of reaction is in linear relationship with the concentration of the enzyme.

As Fig. 2 shows, this dependence could be observed on addition to the samples of 0.01, 0.03 and 0.09 units of activity of PFK: the rate of glycolysis increased proportionately to the added PFK.

The accelerating action of PFK thus obeyed the general rule of enzyme reaction kinetics.

In these experiments a preparation of PFK obtained by the authors' method was used; as noted previously this preparation is homogeneous in the ultracentrifuge [4] but is not quite uniform in enzyme tests; it contains a small

admixture of myokinase [4, 5] and, as was subsequently found, also a small admixture of aldolase. Therefore it was desirable for comparison, to make use also of another PFK preparation. Unfortunately, we could not use the crystalline preparation of PFK as obtained by Gulyi, Dvornikova and Popadiuk for this purpose, since these authors stated that their enzyme consisted of crystalline protein fraction 0.45 and crystalline myogen A [26]; for its maximal molar activity a 4-5-fold excess of myogen A is required over the fraction 0.45 [27]. If it is taken into account that myogen A crystals include: aldolase [28], glycerophosphate dehydrogenase [29] and triosephosphate isomerase [15], and even if it is assumed that the 0.45 fraction is homogeneous (which has not as yet been proven [27]) the Gulyi enzyme preparation still consists of four different proteins. The use of such a complex protein mixture would make it impossible, in the event of a positive result, to establish which of the proteins affected the rate of glycolysis. In view of this, we decided to use the amorphous but highly purified PFK preparation obtained by Ling, Byrne and Lardy, and described in detail by these authors, in 1955 [15]. This PFK preparation is entirely free from myokinase and aldolase admixtures and shows a higher number of turnovers than our preparation; it is therefore eminently suitable for comparison with the action of our preparation.

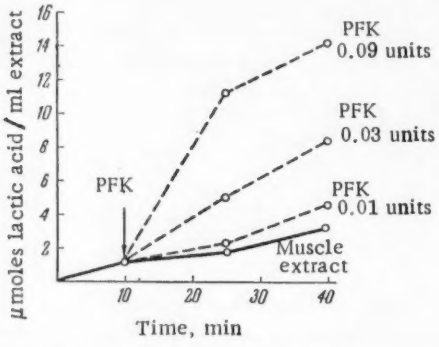

Fig. 2. Dependence of the rate of glycolysis on the concentration of PFK.
Substrate) glycogen.

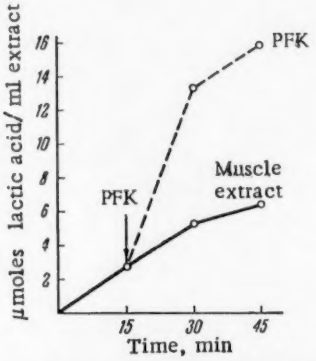

Fig. 3. Rate of glycolysis on addition of PFK (Ling, Byrne and Lardy method): PFK 0.03 units of activity. Substrate) glycogen.

Our experiments on the dynamics of the action of the Ling, Byrne and Lardy PFK showed that its effect was identical to that obtained with our PFK preparation (Fig. 3).

This removed the doubt that the observed increase in the rate of glycolysis might not be due to the action of PFK itself but to that of some other enzyme present as an admixture. But, if extrinsic PFK in fact exerts such an effect, then it follows that muscle PFK is the enzyme which limits the maximal rate of glycolysis. This, however, left the question as to how specific this effect might be and whether some other enzymes might not also possess the ability to increase the rate of glycolysis in the reconstructed enzyme system of muscle, unanswered.

Investigation of Other Enzyme Links in Glycolysis

As the "narrow link" of glycolysis becomes apparent on "extension" of this link through acceleration of the rate of glycolysis, it is possible to reach the reverse conclusion on the absence of influence of a given enzyme on the rate of glycolysis if its addition produces no acceleration of the reaction.

We started the investigation of other links in glycolysis from the central oxidation-reduction reaction which has long been considered by many authors as the limiting factor in the rate of glycolysis [8, 30, 31].

To parallel samples of muscle extract TPD was added in one case and PFK in the other; these additions were made on the 15th minute of glycolysis and the subsequent course of reaction was followed in these samples and in control samples of extract to which no enzymes were added. Despite the fact that 1½ times more TPD was added in terms of activity than of PFK, no acceleration of glycolysis was elicited by the former (there was even slight slowing of the process), whereas the addition of PFK was associated with a marked rise in the rate of glycolysis (Fig. 4).

This raised the doubt that the absence of TPD's effect might have been caused by a deficit of coenzymes

resulting from the addition of a large concentration of TPD. In order to discover the true limiting link another control experiment was set up.

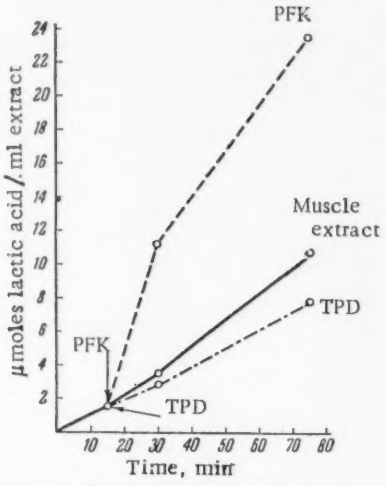

Fig. 4. Rate of glycolysis on addition of TPD.
Substrate) glycogen.

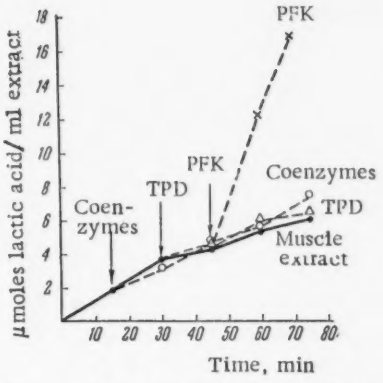

Fig. 5. Rate of glycolysis on addition of coenzymes TPD, PFK:
TPD 0.02 units of activity; PFK-0.025 units of activity. Substrate) glycogen.

To three samples of extract, on the 15th minute of glycolysis, all the coenzymes were added which made up the composition of the reaction medium in amounts calculated to double their concentration. On the 30th minute of glycolysis, TPD was added to two of these samples and on the 45th minute PFK as well was added to one of these samples.

Figure 5 shows that neither the addition of the coenzymes nor the addition of TPD against the background of doubled concentration of coenzymes affected the rate of glycolysis, but that addition of PFK under the same conditions immediately produced a sharp increase in the rate of the reaction.

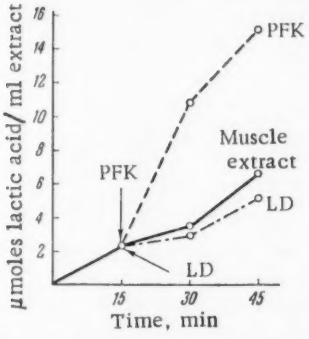

Fig. 6. Rate of glycolysis on addition of LD:
LD-0.033 units of activity; PFK–0.025 units of activity. Substrate) glycogen.

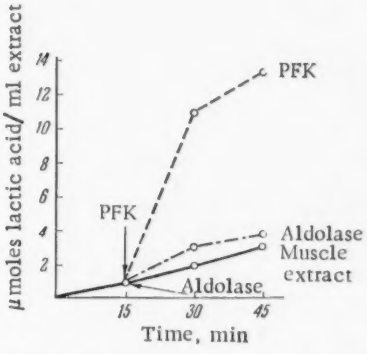

Fig. 7. Rate of glycolysis on addition of aldolase:
Aldolase-0.24 units of activity; PFK–0.025 units of activity. Substrate) glycogen.

Consequently, the possibility of the influence of TPD on the rate of glycolysis in muscle extract was completely excluded.

At one time it had been suggested [3] that another oxidation-reduction process in glycolysis, the reaction catalyzed by LD, might limit the rate of the process. Analogous experiments were therefore staged with LD and with PFK. The results obtained were similar to those of the preceding series (Fig. 6).

The third reaction which had also been implicated in the rate of glycolysis – aldolase reaction – was also

subjected to our tests under similar experimental conditions. The results obtained in this case also proved to be positive for PFK and negative for aldolase (Fig. 7).

The Effect of HK on the Rate of Glycolysis

Carbohydrate breakdown in skeletal muscle is known to involve chiefly glycogen as the initial substrate. However, glucose too is constantly broken down to a small extent [25]. During strenuous work the proportion of direct glucose decomposition apparently increases. This raises the question as to whether the rate, in this case, becomes limited by the HK-reaction, the first stage from which glucose breakdown starts, rather than by PFK.

TABLE 3

Effect of HK on the Rate of Glycolysis

(Composition of reaction mixture as usual. Addition of HK to all samples 25 μg protein. Incubation 60 minutes at 37°)

Initial substrate	Concentration of muscle extract	Lactic acid formation in μmoles per 1 ml extract	
		Muscle extract (without addition)	Muscle extract with added HK
Glycogen	Dilution 1 : 120	2.1	3.5
F-6-P	Dilution 1 : 100	2.3	2.4
Glucose	Without dilution	3.3	48.3

We had already obtained indirect confirmation of such a possibility earlier on the basis of the fact that the power of HK proved to be much less than that of PFK [6] and consequently, of a whole series of other muscle enzymes involved in glycolysis [5]. In the present investigation we also encountered facts which indicated relatively feeble ability of muscle extract to decompose glucose as compared to glycogen breakdown, facts which confirmed indirectly the low activity of muscle HK. Thus, for example, a linear course of glycolysis with glucose substrate was observed immediately upon the most minimal dilutions of the extract, whereas glycogen or F-6-P breakdown required dilution of the extract 40, 80-fold or more to achieve this (Tables 2 and 3). Direct solution of this question could only be provided by experiments with addition of purified HK. This was particularly interesting because the discovery of HK itself by Meyerhof in 1927 occurred under these very circumstances, when addition of a yeast preparation caused acceleration of the fermentation of sugars in muscle extract. Meyerhof at first interpreted these data not as the discovery of a new enzyme but as discovery of an "activator" for muscle glycolytic enzymes [32].

Experiments with highly purified HK were set up similarly to the preceding experiments with PFK, using various substrates for glycolysis.

As can be seen from Table 3, addition of HK exerted no effect on the rate of glycolysis when glycogen and F-6-P were used. On the other hand, when glucose formed the substrate, the addition of the same amount of HK increased the rate of glycolysis more than 14-fold! It must be noted that the effect of HK, as a rule, was more pronounced than that of the addition of PFK despite the fact that both the enzymes were added in approximately the same quantities in terms of units of activity.

This sort of experiment can give rise to doubts as to whether there is a true acceleration effect or whether the enzyme is in some way inactivated in muscle extract and its addition simply restores the activity of the extract.

Meyerhof ascribed the feeble ability to ferment sugars to "ageing" of the extract, understanding by this, inactivation of the enzyme during its preparation, dialysis etc. [32]. Under modern conditions, when all the operations involved in the preparation of muscle extract are carried out in the cold, with the observance of many precautions, it is possible to obtain individual enzymes of high activity, and the concept of "ageing" of the extract in the course of a few hours appears unlikely. Nonetheless we carried out control experiments comparing the action of HK and PFK on the usual dialyzed extract and on perfectly fresh muscle extract.

These experiments showed that before the addition of the extrinsic enzymes, lactic acid was formed more rapidly in freshly prepared extract than in a dialyzed one, i.e., some lowering of glycolytic activity did in fact occur during dialysis. However, after addition of equal amounts of HK to the two samples of extract the rate of glycolysis immediately increased, both in the dialyzed and the freshly prepared extract by more than 10 times, reaching approximately the same level in both samples (Fig. 8, A). When PFK was added, increase in the rate of glycolysis was also observed both in the dialyzed and the fresh extract, although the effect was a bit weaker than that observed on addition of HK (Fig. 8, B).

Consequently, the effect observed by us occurred, not as the result of replacement of inactivated enzyme, since no inactivation could have occurred in the freshly prepared extract, but resulted from actual summation of the weak activity of muscle enzyme and high activity of the same enzyme added extrinsically.

Our experiments thus demonstrated two "narrow" links in glycolysis, two critical stages determining its rate of reaction: the PFK and HK reactions. The final proof evidently had to be an experiment which would demonstrate the two links simultaneously.

With this aim we attempted to accelerate glucose breakdown by means of PFK. The preceding experiments showed that such an effect was impossible (Table 2). We now decided to alter the course of the process by introducing first HK and then PFK into the reaction.

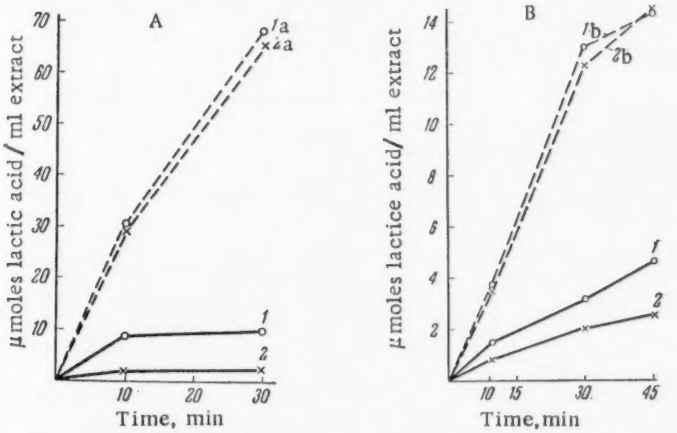

Fig. 8. Effect of HK (A) and PFK (B) on glycolysis in freshly prepared and in dialyzed extract:
1) freshly prepared extract; 1a) the same + HK; 1b) the same + PFK;
2) dialyzed extract; 2a) the same + HK, 2b) the same + PFK.
HK–0.06 units of activity; substrate) glucose. PFK–0.4 units of activity; substrate) F-6-P.

As can be seen from Fig. 9, our attempt proved successful. Owing to addition of extrinsic HK the "narrow" link was "displaced" from the HK-reaction to the other stage, i.e., the PFK reaction. As a result of this, addition of extrinsic PFK accelerated glycolysis, despite the fact that the initial substrate was glucose and not glycogen. Evidently, other enzymatic stages of glycolysis have a higher rate and therefore do not limit the summated rate of the process.

DISCUSSION

There is no need to reiterate the various problems of chemical dynamics of the cell. To study this it would be important to know the way in which the rate of glycolysis in the cell is fixed at one or another level. However, no final hypothesis exists concerning this problem; some of the theoretical concepts are ill-defined and the experimental approach has been very limited (see, for example, the survey made by Kolotilova [34]). Does the rate of glycolysis always depend on the same factor or can different factors limit the course of glycolysis when it is proceeding at different rates? This question has not been posed in its alternative form in the literature since it

has usually been tacitly assumed that only the first possibility was real. This cannot be agreed to however, on the following grounds.

It is known that in intact cells in a state of relative rest the power of the glycolytic enzymes, their potential activity, is utilized to only a very small degree. Calculation shows that the power of even the slowest glycolytic enzyme, expressed in comparable values of Q (μliter gas/ mg dry weight of tissue per hour), is many times higher than anaerobic glycolysis in the tissue from which this enzyme has been isolated [35, 33, 5]. Consequently, even under anaerobic conditions, when the Pasteur inhibition of glycolysis cannot manifest itself, the power of the enzymes in resting tissue is strictly limited by some factor and is utilized only to a small extent. It is clear that in the tissues of an organism relatively at rest, the power of the enzymes cannot limit glycolysis, and this role must be played by another factor.

The situation is different during strenuous work when all the cellular energy reserves are brought into play and the glycolysis Q increases hundreds and thousands of times [1, 8]. There is every reason to believe that in this case the upper limit of the rate of glycolysis is indeed determined by the power of the enzymes. This is confirmed by the close correspondence noted by us between the hourly rate of glycolysis in rabbit muscle in situ during tetanic contractions and in rabbit muscle extract fortified by addition of coenzymes, stabilizers etc. (see Experimental section). This correspondence is not fortuitous.

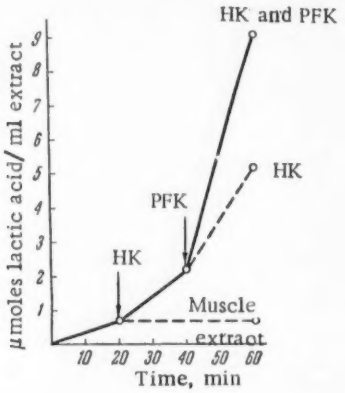

Fig. 9. "Displacement" of the "narrow" link from the HK to the PFK reaction: HK-0.06 units of activity; PFK-0.06 units of activity; substrate) glucose.

The maximal rate of glycolysis in muscle extract where optimal conditions had been created for enzyme action reaches approximately the same level as in the organism as a whole during strenuous muscular work, when, owing to the action of regulatory mechanisms the concentration of carbohydrate and coenzymes does not limit glycolysis [10].

It follows that in the organism the maximal rate of glycolysis depends only on the power of the enzymes.

It must therefore be acknowledged that at rest and during cellular work the rate of glycolysis is determined not by one and the same factor but by different factors.

Some questions of experimental method require comment. If there are two different mechanisms, two factors affecting the rate of glycolysis, it is obvious that their investigation must be effected by different means. As far as factors limiting the rate of glycolysis at rest or during moderate work are concerned, the methods of studying them have only barely been traced. The maximal rate of glycolysis is more amenable to investigation.

For studies of the maximal rate of glycolysis conditions must be created which would be optimal for enzyme action, guaranteeing that during the experiment the same limiting factor as that operating in the body, viz., enzyme power, would be reproduced. This condition is best fulfilled by tissue extract, e.g., Meyerhof's muscle extract. It is important to fortify the extract by addition of necessary coenzymes, activators and stabilizers, so that not these substances, but only the strength of the enzymes would limit the rate of glycolysis in the system. The material commonly used in biochemical investigations – macerate tissue, various homogenates or erythrocyte hemolyzates – cannot by itself, without fortifying additives, be recommended under any circumstances for studies of glycolysis kinetics. The attractive aspect of these preparations, viz., the presence in them of various endogenous factors, is invalidated by the fact that they also contain factors which lower the activity of glycolytic enzymes: very active nucleotidases and phosphatases which quickly destroy coenzymes and also systems which oxidize thiol compounds. It is this fact that is the cause of the very low level of hourly glycolysis in macerated muscle, noted above, compared with stabilized muscle extract. Limitation of the rate of glycolysis in macerated tissue and in homogenates occurs most probably at the stage of central oxidation-reduction owing to the coenzyme and TPD free thiol group deficit which occurs during treatment of the tissue, since it is at this particular stage of glycolysis that the kinetics of the process depend on their concentration to the greatest extent.

In 1940, when little was known about the nature of the glycolytic enzymes, Belitser postulated that it was the central oxidation-reduction that played the role of the "narrow" link in muscle glycolysis [8]. Later, approximately the same concept regarding glycolysis in erythrocytes was formulated by Kolotilova [30]. In recent years a number of authors [36, 31] observed accumulation of F-1, 6-P during glycolysis in macerated muscle, which would also appear to indicate that the limitation of the rate of glycolysis occurred not at the stage of the PFK-reaction but at the oxidation-reduction stage.

Nevertheless, it is quite possible that these observations did not reflect the in vivo kinetics of glycolysis. Just as there is never any appreciable accumulation of F-1, 6-P in the tissues of the organism, so oxidation-reduction is not the "narrow" link in glycolysis in the intact organism, but becomes such a link only as the result of disturbance and impairment of enzyme systems. However, if the reaction medium contains sufficient concentrations of coenzymes (Figs. 5 and 6), both the oxidation-reduction reactions – TPD- and LD-reactions – in no way limit the rate of the process. Since during strenuous muscular work the concentration of the substrate and coenzymes of glycogen breakdown in the cell is maintained by intensive resynthesis at a sufficiently high level [10], these substances cannot limit the rate of glycolysis during strenuous work performed by the body.

During preparation of muscle extract it is impossible to exclude a priori the possibility of impairment and lowering of HK and PFK activity. If this did actually take place, it could give rise to the objection similar to that made by us with respect to experiments with macerated muscle. However, this possibility was completely excluded by direct experiments in which a comparison was made between the activities of freshly prepared and dialyzed extracts (Figs. 8 and 9). Experiments with glucose breakdown showed that even in fresh undialyzed extract containing both dissolved and structurally fixed HK [37] the power of this enzyme was so small that it limited the rate of glycolysis. Analogous results were obtained in experiments with PFK.

Our views concerning the influence of PFK on the rate of glycolysis met with opposition from Gulyi, Dvornikova and Popadiuk [27]. These authors found admixtures of myokinase in the PFK preparation prepared by our method, which was in accordance with our own observation reported in detail [4, 5]; they also found admixtures of aldolase which we had noted too, but after publication of our findings. Without carrying out quantitative determinations of the myokinase and aldolase content, Gulyi et al. nonetheless affirmed that the low power of PFK found by us was not an inherent property of this enzyme but was explained by the insufficient purity of the preparation. At the same time, our paper [4] contained data on the progressive growth of specific activity of PFK on fractionation of muscle extract, and on 50-fold concentration of PFK, as well as a diagram of the course of sedimentation in the ultracentrifuge of the protein obtained by us after purification. This diagram shows only one peak. Since the sedimentation constant S for PFK, myokinase and aldolase equals 6.88 [5] 20,*2.30 [38] and 8.27 [39] respectively, i.e., differs sharply in each case, the presence of a single peak in the diagram indicates that the admixture of myokinase and aldolase to our protein amounts to a concentration below the sensitivity threshold of the ultracentrifuge; in other words it does not exceed 1-2%. Only owing to the greater sensitivity of enzymatic analysis compared to that of the ultracentrifuge can the small admixture of myokinase and aldolase be detected in our PFK preparation. It is extremely doubtful that the crystalline phosphohexokinase of Gulyi which exhibits high activity, according to that author's data [26, 27], only in the presence of crystalline myogen A, i.e., in a system consisting of not less than four different proteins, can be described as a more homogeneous preparation than our PFK. Further, Gulyi, without attempting to compare the activity of PFK with the course of glycolysis, asserts that PFK is not inferior in activity to other glycolytic enzymes and therefore does not limit the rate of glycolysis in muscle. All the material presented by us indicate the opposite. This is particularly apparent from experiments with PFK purified by the Ling, Byrne and Lardy method [15] which contains neither myokinase nor aldolase and which also caused marked acceleration of glycolysis in muscle extract (Fig. 3). On the basis of our previous data and our new observations we thus tend to consider Gulyi's objection unfounded.

Not only our own observations, but also the fact that hexose monophosphate is the only metabolite of glycolysis which is contained in tissues in amounts sufficient for preparative isolation, while F-1, 6-P is contained in barely perceptible quantities, convince us that the limitation of the rate of glycolysis occurs at the stage preceding the formation of F-1, 6-P. The experiments described in the present work indicate directly that such critical stages are the HK and PFK-reactions. In all probability the maximal rate of glycolysis is limited by the power of PFK in those tissues where the predominant substrate for glycolysis is glycogen (skeletal muscle). However, in those tissues in which the main glycolytic process involves glucose breakdown (nerve tissue, heart, erythrocytes, tumors) the maximal rate is determined by the power of HK. The fact that glycogen synthesis in muscle also occurs with the participation of HK does not in any way contradict this hypothesis. It is known that synthesis and

* As in original.

breakdown of glycogen are uncoupled in time, occur at different phases of muscular contraction and proceed at different rates.

The concepts which are being developed by us with regard to "narrow" links in glycolysis lead logically to speculation concerning the means of regulation of cellular metabolism. Every regulatory influence in the cell is reduced, in the final analysis, to directed change in the rate of chains of chemical reactions involved in metabolism. Consequently, demonstration of "narrow" links in glycolysis is also a demonstration of the points of action of the central regulatory influences on the course of energy metabolism [14, 40].

SUMMARY

It is assumed that rates of glycolysis may be determined by different factors under varying functional conditions of the cell and varying levels of glycolysis. The maximal level for rates of glycolysis in muscle in situ (about 1500 μmole lactic acid/gram/hour) can be reproduced in vitro by means of a reconstructed system consisting of: dialyzed muscle extract, ATP, DPN, Mg, F-1, 6-P, orthophosphate, nicotinamide, cysteine and glycogen. When the rate of glycolysis follows a linear course, the "slowest" enzyme of glycolysis may be identified directly by the acceleration of the process which occurs on introducing the purified enzyme. It is shown that none of the enzymes – aldolase, 3-phosphate dehydrogenase or lactic dehydrogenase, hitherto believed to be the slowest enzymes of glycolysis act as such in reality when their activity is displayed in a medium with sufficient coenzyme content. This is the condition prevailing in the body when muscle work is highly active. Depending on the value of 6-phosphofructokinase power, a 4-to 8-fold increase in the rate of glycogen disintegration follows, when purified PFK is introduced. Linear growth of the effect accompanies increasing increments of PFK. The rate of glucose disintegration may even increase to 10-14 times the original value when purified HK has been introduced. The same result has been obtained with a fresh, nondialyzed muscle extract.

It is suggested, that while the rate of glycolysis in skeletal muscle does depend upon PFK power, it is a function of HK in nervous tissue, heart, erythrocytes and tumors.

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THE NATURE OF METABOLISM IN CULTURES OF MONKEY KIDNEY TISSUE

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Various kinds of tissue culture in vitro are used at the present time for raising viruses [1]. The problem of what metabolites and energy sources are required for virus reproduction can be solved only if we know the metabolism of the cells in which the viruses multiply.

The purpose of this work is to study some of the metabolic processes in cultures of monkey kidney cells which are used for raising poliomyelitis virus.

Monkey kidney in vivo is resistant to poliomyelitis virus. Therefore we were interested in the difference between the metabolism of tissue explants and the original tissue.

METHODS

To obtain a suspension of isolated cells, the cortex layer of Macacus rhesus kidney was treated with a trypsin solution by the method of Youngner [2] which has been somewhat modified in our Institute. The suspension of cells, free from intercellular substances, was centrifuged in a refrigerated centrifuge at 4° and about 1500 g. The resulting deposit was transferred to a sterile glass flask which contained Medium No. 199, consisting of a mixture of amino acids, carbohydrates, vitamins, nitrogenous bases, mineral salts, etc. [3]. Two percent of beef serum was added to this nutrient medium. The cells were incubated at 37° for 7-12 days. On the day before biochemical study, the medium was changed to Medium No. 199 without the serum. A solution of trypsin was used to remove the cells of the kidney tissue culture from the glass surface; the loosened cells were centrifuged in the refrigerated centrifuge under the previous conditions, twice washed with physiological saline, and used for the study.

The material with which the tissue culture was compared was a suspension of isolated cells obtained directly after treating the kidney cortex with trypsin and which were twice washed with physiological saline (hereafter called the original tissue).

Tissue respiration was studied by the direct method of Warburg. The intensity of glycolysis was estimated by the accumulation of lactic acid [4].

For study of the rate of succinic dehydrogenase and hexokinase reactions, we used a suspension obtained by homogenizing the cells in the cold for 3 min in a MSE homogenizer with rotation of the knives at 11,000 rpm. The tissue for the determination of the rate of succinic dehydrogenase reaction was homogenized in $5 \cdot 10^{-4}$ M Versene solution, and that for study of the rate of the hexokinase reaction, in 0.12 M phosphate buffer with pH 7.8, which contained 0.15 M NaF. The rate of the succinic dehydrogenase reaction was determined by the intensity of reduction of neotetrazole chloride* [5, 6]. Fixation of the samples was carried out with a mixture of different valumes of alcohol and ethyl acetate. The calibration curve was plotted with synthetic neobiformazane. The optical density was measured at 530 mμ. The rate of the hexokinase reaction [7] was estimated by the decrease in glucose, determined by the Nelson method [8].

Since the content of desoxyribonucleic acid (DNA) in cells is constant [9] and it is possible to calculate the

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number of cells in a tissue culture by the amount of DNA [10, 11], estimation of the metabolic properties was carried out per 1 μg of DNA phosphorus. The DNA was separated from the rest of the phosphorus fraction by the method of Davidson, Leslie, and White [12]. Inorganic phosphorus in the DNA hydrolyzate was determined according to Berenblum and Chain [13]. All the photometric measurements were carried out on a Coleman spectrophotometer (Model 14) or on an Engel photoelectric colorimeter.

Statistical treatment of the results was carried out by the standard method [14].

RESULTS

At first we tried to establish the oxygen requirements of the cells which had been treated with trypsin but not cultured in vitro and of the explanted kidney tissue.

TABLE 1

Oxygen Consumption of Cells of Cultured Tissue and Cells of Original Tissue in the Presence and Absence of Glucose

Gas medium—air; temperature 37.5°; length of incubation—1 hour. Incubation mixture: Ringer-phosphate buffer, pH 7.3—2.4 ml, 5% glucose—0.1 ml, cell suspension—0.5 ml. In the sample without glucose, 0.9% NaCl—0.1 ml. Values expressed in μliters O_2 per 1 μg DNA phosphorus

Without glucose	In presence of glucose	Change in respiration in glucose sample in %	Without glucose	In presence of glucose	Change in respiration in glucose sample in %
Cultured tissue			7.2	5.5	−23.6
4.2	2.9	−31.0	7.9	4.9	−37.9
3.2	2.0	−37.5		Average	−26.63±3.48**
1.7	1.0	−41.2		Original tissue	
5.0	3.4	−32.0			
5.4	4.3	−20.4	3.2	5.9	84.4
2.1	1.7	−19.0	3.8	4.3	13.2
5.1*	2.8*	−45.1	1.3	2.3	76.9
2.2*	2.2*	0	1.6	2.4	50.0
3.5*	2.7*	−22.9	1.5	2.7	80.0
6.7	4.1	−38.8	2.1	2.2	4.8
8.0	6.8	−15.0	6.7	7.9	17.9
4.4	3.9	−11.4	9.7	12.0	23.7
6.2	5.3	−14.5		Average	43.86±11.67
7.0	4.5	−35.7			

In these experiments the cells were removed from the glass surface with a 0.02% Versene solution in a Diul'bekko buffer without addition of $CaCl_2$ and $MgCl_2$.

**In this table and in all the following ones we give the arithmetical average (M) with the mean error (m). The change in respiration under the influence of glucose in the cultured tissue ($t = 8.37$; $p < 0.001$) and in the original tissue ($t = 3.76$; $0.01 > p > 0.001$) is statistically significant.

As table 1 shows, kidney tissue cultures are entirely different from the original cell suspension. While glucose stimulates respiration in the monkey kidney cells treated with trypsin, it produces an inverse Pasteur reaction in the explanted tissue [15], that is, respiration is suppressed in the presence of glucose. The existence of the inverse Pasteur effect in cultures of kidney tissue obviously indicates a definite change in the interrelation of respiration and glycolysis which occurs during growth in vitro during a relatively short period of time.

In order to extend our understanding of the oxidizing mechanism of the tissue culture cells we studied the oxygen requirements of these cells in the presence of p-phenylenediamine and succinate.

Table 2 gives the results of these experiments compared to the data obtained in a study of the original kidney cells. The slowing of the rate of oxidation of p-phenylenediamine by the tissue culture cells evidently shows

*Transliterated from Russian.

an injury to the cytochrome portion of the oxidation chain. The lack of increased respiration in the presence of succinic acid could be due to the injury which we observed in the cytochrome system, or to a decreased rate of the succinic dehydrogenase reaction.

The results given in Table 3 show that the rate of the succinic dehydrogenase reaction in the tissue culture cells is considerably less than the rate of this reaction in the original tissue.

TABLE 2

Oxygen Consumption of Tissue Culture Cells and Cells of the Original Tissue in the Presence of p-Phenylenediamine and Succinic Acid

In μliters O_2 per 1 μg DNA phosphorus. Incubation mixture: Ringer-phosphate butter, pH 7.3 – 2.4 ml; 0.33 M p-phenylenediamine or 0.28 M Na succinate – 0.1 ml; cell suspension – 0.5 ml. The samples without substrate had a corresponding amount of 0.9% NaCl. Conditions of incubation the same as in Table 1

Tissue culture				Original tissue			
Control	p-Phenyl-enedi-amine	Na suc-cinate	% increase in respira-tion	Control	p-Phenyl-enedi-amine	Na suc-cinate	% increase in respira-tion
7.5	14.8	—	97.3	2.3	10.3	—	347.8
4.8	6.8	—	41.7	1.4	14.8	—	957.1
5.2	7.1	—	17.5	1.6	8.6	—	437.5
			52.17±23.55				580.80±60.05
4.4	—	5.3	20.5	1.6	—	8.2	412.5
3.9	—	5.2	33.3	2.6	—	9.6	269.2
2.1	—	2.4	14.3	2.1	—	8.3	295.2
7.0	—	8.8	25.7				325.63±34.17
4.0	—	4.0	0				
			18.76±4.71				

Note: The difference between the tissue culture and the original tissue for stimulation of respiration by p-phenylenediamine ($t = 8.19$; $0.01 > p > 0.001$) and Na succinate ($t = 8.89$; $p < 0.001$) is statistically significant.

When we had established the fact of the injury to the cytochrome and succinic dehydrogenase portions of the oxidation mechanism in tissue explants, we attempted to determine whether the tissue culture cells were potentially able to carry out a supplementary oxidation of glucose and endogenous substrates when artificial hydrogen acceptors were present in the medium. We used methylene blue as such an acceptor. Table 4 shows that in the presence of methylene blue there was an increase in endogenous respiration. In the tests with methylene blue, glucose stimulated the oxygen consumption and did not suppress it. Direct determination showed that methylene blue increased the utilization of glucose, but did not increase the accumulation of lactic acid. This agrees well with our previous study [16], which has been confirmed in other laboratories [17] and which showed that methylene blue decreased the inverse Pasteur reaction and increased the supplementary consumption of glucose. Thus, these data show that the hindrance to respiration in the tests with glucose (inverse Pasteur reaction) is related to competition between respiration and glycolysis for the total hydrogen acceptor.

The damage which we found in the cytochrome and succinic dehydrogenase systems may play an important part in this.

In addition to the oxidation processes, we also studied the intensity of aerobic and anaerobic glycolysis in monkey kidney cells. The results of our experiments indicated the significance of activation of aerobic glycolysis during growth of kidney cells in vitro (Fig. 1).

It is known that injury to the cells can cause activation of aerobic glycolysis [18]. It may be that in our experiments the high aerobic glycolysis in the tissue culture cells is caused by factors such as trypsin, inadequacy of the medium, or centrifuging. However, the relatively low level of aerobic glycolysis in the original tissue, which

was subjected to the same factors, contradicts this idea. It is true that the low level of aerobic glycolysis in the suspension of cells which was not cultured in vitro might be due to some irreversible changes which occurred during the action of trypsin on the kidney, an action which lasted for several hours.

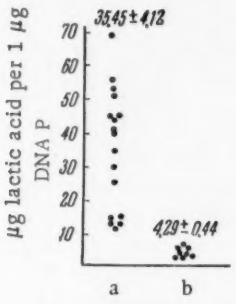

Fig. 1. Formation of lactic acid by tissue culture cells (a) and cells of original tissue (b) under aerobic conditions. Values on graph – Arithmetical average (M) and mean error (m). Difference in intensity of aerobic glycolysis between tissue culture and original tissue is statistically significant ($t = 7.53$; $p < 0.001$).

In order to eliminate this objection, we kept the tissue culture cells in a moist chamber at 37° without addition of nutritive medium for 4 hours, and then incubated them in the cold with a trypsin solution for 2 hours. The amount of lactic acid formed in the cells (expressed per 1 μg DNA phosphorus) in two experiments was 40.5 and 15.1 μg, and in parallel studies on normal cells was 44.0 and 15.3 μg. Thus these factors have no effect on the level of aerobic glycolysis.

We should remark that our special experiments showed the absence of any appreciable loss of lactate during the incubation period either in the tissue culture or in the original cell suspension. This permits us to estimate without any great error the intensity of aerobic glycolysis by the amount of lactic acid formed during incubation.

The data of Table 5 indicate that there is also a high level of anaerobic glycolysis in kidney tissue cultures compared to glycolysis in cells treated with trypsin but not cultured in vitro.

One of the explanations for the increase in glycolysis in tissue explants may be a change in the activity of specific enzymes in the organism, which are controlled by the action of various humoral factors. According to data obtained in our laboratory, monkey kidney tissues undergo glycolysis in which glucose is the main substrate. Thus, the limiting factor in glycolysis in our cultures is hexokinase [19, 20].

TABLE 3

Succinic Dehydrogenase Activity in Tissue Culture and Original Tissue

In μg neobiformazane per 1 μg DNA phosphorus. Gas medium – air; temperature 38°; length of incubation – 40 min. Incubation m mixture: 0.1 M phosphate buffer, pH 7.4 – 1 m.; 0.2 M Na succinate – 0.5 ml; 0.5% neotetrazole chloride – 0.2 ml; 0.2 % KCl – 0.1 ml; 0.9% NaCl – 0.2 ml; homogenate 1 : 10 – 1 ml. In the control experiments instead of Na succinate we used the corresponding amount of 0.9% NaCl. The endogenous controls are deducted from the data.

Tissue culture	Original tissue
3.3	9.6
3.2	7.5
2.5	14.5
5.5	11.5
1.5	12.3
Average 3.2±0.97	11.08±1.18

Note: The difference in succinic dehydrogenase activity of the tissue culture and original tissue is statistically significant ($t = 5.15$; $p < 0.001$).

The data in Table 6 show that the rate of the hexokinase reaction in kidney tissue cultures is much greater than in the original cell suspension.

DISCUSSION

The most interesting fact which emerges from the results which we have obtained is that monkey kidney tissue cells, after a very short period of culturing in vitro, show metabolism which is not characteristic of the original material. The literature data also indicate changes in metabolism [18, 21-24] and in morphology (see, for example [25, 26]), which occur when cells are cultured in vitro.

The monkey kidney tissue cultures which we studied show some similarities in metabolism to malignant tumors. The presence of intensive aerobic and anaerobic glycolysis, damage to the system which oxidizes succinic acid and p-phenylenediamine – all these are characteristics of cancer tissue [27]. We should also remember the presence in our explants of the inverse Pasteur reaction which has been observed in cultures of Walker sarcoma cells [28], in ascitic cancer cells [16, 29], and in some other tumors.

The question arises as to whether the change in metabolism in our explant tissue is an sign of spontaneous formation of a malignancy. This question is most significant, since the process of malignancy formation in cells

cultured in vitro has now been established. According to the literature, the formation of malignant tumors in vitro is usually connected either with repeated transfer of the cells [30, 31], with special (anaerobic) conditions of growth [32], or with the use of carcinogens [33]. Our cells were grown in vitro for a very short time (7-12 days) and did not undergo the above treatments.

TABLE 4

Consumption of Oxygen by Kidney Tissue Culture Cells in the Presence of Methylene Blue

In μliters O_2 per 1 μg DNA phosphorus. Incubation mixture: Ringer-phosphate buffer, pH 7.3– 2.3 ml; 5% glucose–0.1 ml; 10^{-4} M methylene blue–0.1 ml; cell suspension 0.5 ml. In tests without methylene blue or glucose–the corresponding amount of 0.9% NaCl. Incubation conditions same as in Table 1.

Addition				Stim. of endogenous respir. by methylene blue	Stim. of resp. by glucose in presence of methylene blue
Without addition a	Glucose b	Methylene blue c	Glucose + methylene blue d	$\frac{(c-a)\cdot 100}{a}$ %	$\frac{(d-c)\cdot 100}{c}$ %
8.0	6.8	9.2	12.1	15.0	31.5
4.4	3.9	6.0	7.9	36.4	31.7
6.2	5.3	8.4	10.2	35.5	21.4
				28.97±3.38	28.20±3.56

Note: Stimulation of respiration by methylene blue in the endogenous tests ($t = 8.69$; $0.05 > p > 0.01$) and stimulation of respiration by glucose in tests with methylene blue ($t = 7.93$; $0.05 > p > 0.01$) are statistically significant.

TABLE 5

Formation of Lactic Acid by Tissue Culture Cells and Cells of the Original Tissue under Anaerobic Conditions.

In μg lactic acid per 1 μg DNA phosphorus. Gas medium–nitrogen; temperature 37.5°; length of incubation–1 hour. Incubation medium the same as in Table 1. Values of endogenous controls deducted.

Tissue culture	Original tissue
13.4	2.3
33.9	2.5
51.8	3.0
75.0	1.0
Average 43.5±13.13	2.2±0.36

Note: Difference in anaerobic glycolysis level for tissue culture and original tissue is statistically significant ($t = 3.11$; $0.05 > p > 0.01$).

TABLE 6

Rate of Hexokinase Reaction in Tissue Culture and Original Tissue

In μg loss of glucose per ρ μg DNA phosphorus. Gas medium–air; temperature 30°; length of incubation 8 min. Incubation medium (final concentration): Na phosphate buffer, pH 7.8–$4 \cdot 10^{-2}$ M; glucose $2.4 \cdot 10^{-3}$ M; KCl–$4.2 \cdot 10^{-2}$ M; $MgCl_2$–$5 \cdot 10^{-3}$ M; NaF– $5 \cdot 10^{-2}$ M; ATP–$5 \cdot 10^{-3}$ M. Homogenate 1 : 2–0.2 m. Total volume 0.5 ml.

Tissue culture	Original tissue
26.0	3.7
21.2	3.8
15.0	1.5
27.0	
20.7	
Average 21.98±2.19	3.0±0.79

Note: Difference in hexokinase activity of tissue culture and original tissue ($t = 8.15$; $p < 0.001$) is statistically significant.

Thus, it is very unlikely that in our experiments we were dealing with malignant tissues. However, we cannot exclude the possibility that the changes in metabolism of the tissue cultures which we observed might supply a favorable basis for later formation of malignancies.

As we showed, the fact of increased intensity of aerobic glycolysis in the tissue culture is not explained by injury to the cells. A study of the formation of lactic acid in the decanted culture liquid [34] also showed a high level of glycolysis in monkey kidney cells grown in vitro. The presence of intense anaerobic fermentation in the explant cells and also the high rate of the hexokinase reaction indicate that the reason for the increased glycolysis in the tissue culture should be sought in the activation of the individual steps in the glycolytic chain which have no relation to injury of the cells. This is indicated by the increased rate of the aldolase reaction in monkey kidney cells cultured in vitro [34].

These facts do not fit the conception of Warburg [18, 35], who considers that high aerobic glycolysis is a specific characteristic of malignant tumors and that normal, intact cells do not undergo glycolysis under aerobic conditions. Aside from the data on intense aerobic glycolysis, for example, in intact leucocytes [36], and in embryonic tissue cultured in vitro [37], the results of our study put Warburg's theory in doubt. We can probably think that all these complex biochemical properties which are considered to be characteristic for malignant tumors [27] can also occur in some normal tissues.

It should be remarked that not all tissues which grow outside the organism show the metabolism which we found for explanted cells of monkey kidneys: the metabolism of various tissues cultured in vitro is not always the same [18, 23, 28, 37-40].

An explanation of the special metabolism found in the reproduction of viruses has great interest. We know that in the latent period in the multiplication of poliomyelitis viruses activation of glycolysis occurs in kidney tissue cultures, and then its inhibition begins [34]. We also know that cellular respiration is an important factor for the formation of new virus particles [41]. It is interesting that the poliomyelitis virus, which usually grows on monkey kidney cell cultures, can also be cultured on explants of many blastomatous tissues.

The question of the significance of the changes in metabolism which we have observed in kidney tissue cultures for the synthesis of virus proteins will be the object of further study.

SUMMARY

Macacus rhesus kidney tissue cells were cultivated in a synthetic medium "199" with 2% bovine serum for 7-12 days. For comparison a suspension of separate single cells obtained from the cortex tissue after treatment with trypsin was used.

Glucose inhibited respiration (inverse Pasteur reaction) in tissue culture cells and increased oxygen utilization in the original kidney cortex cells. Succinic acid and p-phenylendiamine oxidation in the kidney tissue culture was considerably reduced in comparison with the original cells.

Succinic dehydrogenase activity was significantly lower in the culture cells.

Methylene blue increases endogenous respiration and glucose utilization in culture cells without increasing lactic acid accumulation.

Aerobic and anaerobic glycolysis are more intense in culture cells, and the hexokinase activity is high.

The similarity between the metabolic characteristics of tissue cultures and malignant tissues are discussed.

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THE ANTIVITAMIN ACTION OF PLANT EXTRACTS

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Much is known about the suppression of yeast growth by plant extracts and tissues. Information has been accumulated about the inhibition of yeast growth by analogs and derivatives of vitamins and other substances which are classed as antimetabolites. The possibility has not been excluded that plant extracts may contain antagonists to some of the vitamin B group and that these might be detected with the help of some microorganisms which require these vitamins.

This paper presents the results of a study of antagonistic action of plant extracts on biotin and pantothenic acid. In the rest of this paper, substances which have the antivitamin action of the extracts will be called antivitamins.

EXPERIMENTAL

Material and Methods

The basic study was made on materials from three plants: peas (Pisum sativum) of the Shtambov and Amur varieties, beets (Beta vulgaris) of the Bordosskaia variety, and poppies (Papaver somniferum).

The idea of the possible presence of antivitamins in peas and beets is comparatively old. Rippel discovered a substance in pea sprouts which suppressed the development of yeasts of the Saccharomyces type [1]. This substance was extremely specific in its action and showed no inhibitory effects on other types of yeasts or molds. Later, Rippel described some physical and chemical properties of this substance; it decomposed when dried, boiled, or treated with alkali, and was adsorbed by charcoal. It appeared at the start of germination in pea sprouts and was present in growths of 2-3 weeks [2]. Its chemical nature was not established.

We prepared a concentrated extract with high activity from the sap of peas.

Green or etiolated 12-15-day-old plants were ground and the sap pressed out. The protein was precipitated by heat and the filtered sap was treated with activated charcoal. The antivitamin was eluted with 96% alcohol and the alcoholic eluate was evaporated under vacuum to a sirupy consistency. The sirup was cherry-red in transmitted light and contained a considerable quantity of the antivitamin. We obtained 30-50 ml of concentrate from 10 liters of sap; its activity was 200-300 times higher than the activity of the original sap and it showed a marked inhibitory effect when it was diluted 5-6 million times.

The antivitamin molecule was acid, since it was not adsorbed by silica gel and during electrophoresis it moved to the positive pole. The antivitamin lost its activity completely in 5 min in a 0.1 N alkali solution and in 0.1 N $KMnO_4$ for 2 hours it lost 90% of its activity. When the antivitamin was autoclaved for 1 hour in a sealed ampule at 2 atm., it decomposed. At room temperature the activity of the preparation in the ampule lasted for 6-8 months and was not entirely destroyed after keeping for 2-5 years.

In a study of the immunity of sugar beet roots, Oparin and Kuplenskaia [3] found that beet sap suppressed the growth of yeast. They assumed that the substance which suppressed the growth of yeast was a product of intermediate metabolism in the beet root. The authors gave this substance the temporary name "antibios", since they assumed that the action of this substance on yeast took place by inactivation or blocking of the substance bios.

The substance was very unstable; it decomposed in the tissues when they were rapidly cooled, rapidly heated, or dried. Outside the tissues the substance was somewhat more stable. In pressed juice it withstood hot

sterilization and boiling with 1% HCl. After extraction, it was detected in fractions which contained saponin, phosphatide, and phytosterol. A distinct inhibiting action on yeast growth was found when the beet sap made up 10% of the total nutritive medium [4].

In the present work the antivitamin was not added to the medium as sap pressed from the cells, which acts at relatively high concentrations, but as disks of tissue cut from the root under aseptic conditions.

The diameter of the disks was 4 mm, the thickness 2.5 mm, the volume 0.03 ml, which was 0.5% of the volume of the nutritive medium. The disk was washed with sterile water to remove cell fluids which resulted from mechanical injury to the cell surface and was placed individually in tubes with sterile media. Such a disk could be considered a dialyzer immersed directly in the nutritive medium in which the yeast cells grew.

We tested other plants for antivitamin action by noting the effect on yeast growth of aqueous extracts of leaves, stems, and flowers; we used about 150 types of plants in which the biotin content had been determined in the water-soluble fraction [5]. Only extracts from the corolla of the poppy showed an inhibitory action on yeast growth. The extract was prepared from the dried corollas by heating for 30 min in a Koch boiler (1 g of material + + 20 ml of water). The extract was prepared just before use.

We used a strain of yeast (Saccharomyces cerevisiae 77) as the indicator for the antivitamin action of the plant extracts; this had been used for the quantitative determination of biotin [6]. This strain had a definite need for vitamins and grew well on the nutritive medium with the following composition:

$MgSO_4 \cdot 7H_2O$	1.0 g	Sucrose	20.0 g
$(NH_4)_2SO_4$	3.0 g	Asparagine	1.0 g
KH_2PO_4	2.5 g	Thiamine	4.0 mg
$ZnSO_4 \cdot 7H_2O$	1.0 mg	Inositol	10.0 mg
$CuSO_4 \cdot 5H_2O$	0.1 mg	Ca Pantothenate	0.5 mg
$FeSO_4 \cdot 7H_2O$	0.85 mg	Pyridoxine	1.0 mg
		Biotin	0.025 mg.

The mixture was diluted with distilled water to 1 liter. This medium had pH 5.0. If the extract which was to be studied changed the pH of the mixture, NaOH was added to restore the original pH (5.0).

This medium, hereafter called "complete," was added in amounts of 5-7 ml to the tubes to which the different extracts were added. When they were needed, media were prepared without any vitamins or with some vitamins excluded. The tubes were stoppered with cotton plugs and sterilized for 30 min at 115°. The yeast was added to the sterile tubes. The amount of added yeast was controlled nephelometrically. Suspensions in 100 tubes were prepared with small loops of yeast (diameter 3 mm) from 3-10-day-old cultures on wort agar. Incubation was carried out for 24-72 hours, depending on the purpose of the experiment. The amount of yeast added to the tube was determined by the photoelectric colorimeter and was expressed in units of turbidity. In order to obtain the most comparable data we measured the turbidity of a yeast suspension with a known content of dry cells in a unit volume, and for a unit of tubidity we took the turbidity produced by 1 microgram of dry yeast in 1 ml of nutritive solution.

In the tables we give the nephelometrically determined weight in micrograms of dry yeast substance in the tubes (average of five determinations) and these figures express the turbidity of the suspension in relative units.

The yeast strain S. cerevisiae 77 can be used to detect the antagonists of two vitamins – biotin and pantothenic acid, since when either of these is excluded from the nutritive medium, yeast growth is sharply decreased at the beginning of incubation (Table 1).

Table 1 shows that asparagine increases the development of yeast on a nutritive medium without biotin or pantothenic acid. Excluding pyridoxine from the nutritive medium does not affect the growth of yeast. Earlier [6] we showed that this strain is not very sensitive to the absence of inositol and thiamine in the nutritive medium. Thus, this strain can show changes in growth rate when antagonists to biotin and pantothenic acid are present in the medium. The possibility is not excluded that the plant extracts influence yeast growth in some way other than by blocking these two vitamins, but this question will be discussed later.

Antivitamin Action of Extracts from Pea Sprouts

The pea extracts slow the growth of yeast not only on a synthetic medium, but also on wort, which

is the best nutritive medium for yeast. The action of different concentrations of the extract in the medium was contradictory (Table 2).

TABLE 1

Growth of S. cerevisiae 77 in Relation to the Presence of Vitamins

(values expressed in μg of dry yeast weight)

Type of medium	With asparagine			Without asparagine		
	Incubation in hours					
	25	42	68	25	42	68
Complete medium	26	581	768	38	530	776
1750 times less biotin	0	126	195	0	16	83
Biotin excluded	0	10	34	0	0	5
Pantothenic acid excluded	10	224	692	0	123	524
Pyridoxine excluded	0	741	646	26	602	812

TABLE 2

Action of Concentrated Extracts of Pea Sprouts on Yeast Growth

% Content of extract in the medium	Action on yeast growth	Remarks
Up to 0.002	Weak stimulation	Vitamins and amino acids act thus
0.005-0.5	Growth suppressed	Shows antivitamin action
2.0-5.0	Strong stimulation	Rapid yeast growth as on wort alone

The relation of concentration of extract and type of action is not absolute. It depends on the minimum amount of yeast used in the experiment: about 1-15 mg of crude yeast per liter of nutritive solution. The less the amount of yeast used in the experiment, the lower will be the concentration of extract with which inhibition begins. If a gradually increasing amount of yeast is added to the tubes with a constant content of extract, the result is a shift from complete inhibition to rapid growth. The length of incubation is also of importance. After a longer period the yeast begins to grow under conditions of higher concentration of the extract. We found that after complete inhibition for 5-20 days, rapid yeast growth began. When it was kept under standard conditions it gave constant, reproducible results.

TABLE 3

Action of High Concentrations of Extract on Yeast Growth

(values in μg dry yeast weight)

Type of medium	With asparagine				Without asparagine			
	Concentration of pea extract in μl/ ml of medium							
	0	0,8	20	100	0	0,8	20	100
Complete medium	617	0	0	21	457	0	0	15
Excluding biotin	316	0	0	19	85	0	0	13
Excluding pantothenic acid	562	0	0	19	389	0	0	15
Excluding biotin and pantothenic acid	37	0	0	35	19	0	0	28
Excluding biotin and pantothenic acid and adding β-alanine (1 μg/ ml)	28	0	0	19	22	0	0	13

Table 3 shows the action of high concentrations of extract on the threshold of complete inhibition, when further increase in concentration begins to increase the development of yeast.

The experiments include several types of nutritive media with different vitamin content. Differences in yeast weight without any extract are completely determined by the presence of vitamins in the nutritive medium. Minimal and average concentrations of extract suppress growth in all types without exception. At the highest concentrations inhibition is replaced by growth, though it is slow, and this is seen in all types. This growth does not depend on the vitamin composition of the medium; this is partly because of the presence of vitamins in the extract. The introduction of large amounts of concentrated extract equalizes the vitamin content in all types of medium. When asparagine is not added to the medium, yeast growth slows not only in the controls but also above the threshold of inhibition at the highest extract content.

Here we encounter an unusual effect, the same compound having a contradictory action on the same substance, with the result that in small amounts it acts as an antimetabolite and in large amounts has a metabolic function. Only a few substances have similar actions. Among these is α-methylpantothenic acid, an analog of pantothenic acid, which in small amounts suppresses the growth of Lactobacillus casei, and in large ones, stimulates it [7]. The formation of pantothenic acid from the analog is not possible and so the stimulating action is that of the substance itself. It must be remembered that in our experiments we did not use a definite chemical compound, but a plant extract and so a combined action of several substances was possible.

Table 4 shows the action of concentrations below the inhibitory threshold on yeast growth in media which differ in vitamin content.

TABLE 4

Growth of Yeast at Low Concentrations of Pea Extract

(values in μg dry yeast substance)

Type of medium	With asparagine					Without asparagine				
	Concentration of pea extract in μl/ml of medium									
	0	0.001	0.01	0.1	1.0	0	0.001	0.01	0.1	1.0
Complete medium	110	129	78	0	0	105	195	151	240	0
Excluding biotin	45	107	66	0	0	22	22	23	28	0
Excldding pantothenic acid	22	20	13	0	0	28	151	263	209	0
Excluding biotin and pantothenic acid	15	13	15	0	0	23	19	19	17	0
Excluding biotin and pantothenic acid and adding β-alanine (1 μg/ml)	58	66	71	0	0	19	22	22	22	0

In the presence of asparagine the inhibitory action of the extract is increased about tenfold. Full inhibition begins in all types at the same concentration of extract and so it must be that it is the asparagine which exerts the inhibitory action, and not differences in vitamin concentration in the medium. The weight of yeast in the different types of medium differs greatly and the reason is plain: in the five types the yeast weight varied from 13-28 mg. This minimum weight of yeast was found in the absence of biotin or pantothenic acid or in the simultaneous absence of both vitamins from the medium. Growth within these minimum limits was due to intracellular reserves of vitamins. With a partial deficiency of one vitamin, the weight of yeast was 45-70 mg and when all the vitamins were present in available form, the weight reached maximum values (100-260 mg).

The minimum amount of extract stimulated the growth of yeast even in the complete medium; this was because the extract contained vitamins and other substances which favored yeast growth.

The properties of asparagine and aspartic acid which permit them partly to replace biotin in yeasts and some lactobacilli have long been known [8, 9]. Table 1 showed that this also holds for the strain S. cerevisiae 77. Addition of asparagine to the nutrient medium increased the rate of yeast growth at any vitamin concentration in the medium.

In the presence of pea extracts the action of asparagine on yeast growth was partly changed. It kept its

ability to replace biotin and here also yeast grew well in media in which the biotin was replaced by asparagine. However, in the presence of pea extracts, asparagine showed an antagonistic action toward pantothenic acid – it strengthened the action of the antagonists. The connection between asparagine, antagonists, and pantothenic acid was shown by analysis of the yeast growth in different types of media with the same content of extract; for example, 0.00001 ml of extract in 1 ml of medium (Table 5).

TABLE 5

Action of Vitamins and Asparagine on Yeast Growth in the Presence of Pea Extracts

Type of medium	Nutritive medium with pea extract (0.01 µl of extract per 1 ml of medium)	With asparagine		Without asparagine	
		Vitamin composition of medium and yeast growth	Weight of yeast in µg	Vitamin composition of medium and yeast growth	Weight of yeast in µg
1	Complete medium	Ba + B + PAe + A Part of the pantothenic acid is blocked	78	B + PA + PAe All vitamins present in available form. Maximum growth.	251
2	Excluding synthetic biotin	Ba + PA + PAe + A Part of the pantothenic acid is blocked; partial deficiency of biotin	66	PA + PAe Biotin absent. Growth minimal.	23
3	Excluding synthetic pantothenic acid	Ba + B + BAe + A All pantothenic acid of the medium is blocked. Growth minimal.	13	B + PAe All vitamins present in available form. Maximum growth.	263
4	Excluding synthetic biotin and pantothenic acid	Ba + PAe + A All pantothenic acid blocked. Growth minimal.	15	PAe Biotin absent. Growth minimal.	19
5	Excluding synthetic biotin and pantothenic acid, adding β-alanine (1 µg/ml)	Ba + β-alanine + PAe + A Pantothenic acid blocked, but β-alanine partly replaces it. Average growth.	71	β-alanine + PAe Biotin absent. Growth minimal.	22

The biotin content is the sum of synthetic biotin (B), added in the amount of 25 µg/liter and the biotin-like action of asparagine (Ba), which permits the growth of yeast without biotin in the nutritive medium. There is a very small amount of biotin in the pea extract, for during the concentration most of it remains on the activated charcoal. In the dilutions which were used, the biotin of the extract showed no physiological action on the yeast. The pantothenic acid content is the sum of the synthetic pantothenic acid (PA) and the acid in the extract (PAe). A minimum addition of extract replaces the synthetic pantothenic acid and permits maximum yeast growth. Asparagine is designated by the letter A; the blocking action of asparagine and the antagonists is shown by the frames. Growth of yeast above the minimum was possible only in the presence of added biotin (or biotin active substances) and free pantothenic acid.

We can assume that the antagonist acts by blocking small amounts of biotin but only in the absence of asparagine, that is, it blocks biotin and does not affect the biotin-like action of asparagine. All five types of medium seem to confirm this assumption: addition of synthetic biotin to the medium eliminates the action of the antagonist and permits maximum growth of yeast. However, it is more probable that the absence of growth in the types without synthetic biotin results not from suppression of the minimal dose of biotin, but from its absence. In the contrary case of an asparagine medium with synthetic biotin (type 3) yeast should grow more rapidly than on a medium without synthetic biotin (type 2). Actually in type 2, the yeast grows faster.

If we assume that the antivitamin acts by competitive blocking of pantothenic acid in the medium or a pantothenic-acid-like function of the extract, and asparagine only strengthens this action, then all the values in Tables 4 and 5 can be explained without inconsistancy. Asparagine strengthens the action of the antagonist and so maximum growth occurs in media without it. The antagonist competes with pantothenic acid, and so in types 1 and 2 in which supplementary synthetic pantothenic acid is present, yeast grows faster. β-Alanine competes with the antagonist, since it is a precursor in the biosynthesis of pantothenic acid in yeast.

With an increase in concentration of extract, the competitive relation of antagonist and pantothenic acid becomes non-competitive, since the inhibition goes beyond dependence on the level of pantothenic acid in the medium. Asparagine strengthens both the competitive and non-competitive action of the antagonist.

The Antivitamin Action of Substances from Beets and Poppies and Possible Reasons for the Antagonism

The tissues of beets autoclaved with the nutritive medium permit good yeast growth in media without biotin and pantothenic acid. Yeast growth in the controls is considerably slower. We can assume that the antivitamin is destroyed completely or partly by heating. The content of biotin and pantothenic acid in beets has been frequently determined [10-12]. The separate tissue disks which weigh 0.03 g contain about 0.004 μg of biotin and 0.04 μg of pantothenic acid. These amounts of tissue vitamins in 5 ml of nutritive medium permit normal yeast development. Disks of live beet tissue slow the accumulation of the weight of yeast in the culture (Table 6).

TABLE 6

Effect of Vitamins on Yeast Growth in the Presence of Beet Tissue

Type	Composition of nutritive medium	Weight of yeast in μg			Nature of action of live tissue
		Control		Live tissue	
		Dead tissue	Without tissue		
1	Excluding biotin	177	105	167	Increasing
	Excluding pantothenic acid	144	120	95	21% inhibition
2	Excluding biotin	155	63	140	Increasing
	Excluding pantothenic acid	130	70	18	74% inhibition
3	Excluding biotin	132	15	126	Increasing
	Excluding pantothenic acid	202	15	0	100% inhibition

The types differ in amount of yeast added to the medium at the beginning of the experiment: in type 2 we added 5 times less and in type 3, 25 times less than in the first. The less yeast we added in the experiment, the higher the percent inhibition; thus, in type 3 we found complete suppression of yeast growth. Exclusion of biotin from the nutritive medium did not decrease the yeast growth compared to the control without tissue. In all types of medium without biotin, the live tissue permitted maximum yeast growth; hence biotin diffused from it.

TABLE 7

Significance of Pantothenic Acid Concentration in Preventing Inhibition of Yeast Growth by Beet Tissue

Pantothenic acid concentration, in μg	Weight of yeast in μg		% Inhibition
	Control without tissue	Live tissue	
0	13	0	100
0.01	84	74	12
0.02	104	98	10
0.03	136	124	9
0.04	164	148	10

Live beet tissue inhibited yeast growth when pantothenic acid was absent from the nutritive medium. Addition of pantothenic acid to the medium prevented the inhibiting action of the live tissue; that is, pantothenic acid competes with some substance which acts as an antagonist to pantothenic acid. The competition of pantothenic acid and the antagonist is shown in Table 7.

Under the experimental conditions the requirement for pantothenic acid to repress the action of the antagonist is small, about 2% of its content in the complete nutritive medium. A slight inhibition remains and further addition of pantothenic acid has almost no effect.

Live tissue does not absorb pantothenic acid from the solution nor inactivate it in solution. Moreover, solutions of pantothenic acid in which the tissues have been kept contain more pantothenic acid than the control solution which confirms the dialysis of the vitamin from the tissue. We conclude from these observations that the antivitamin is given off in small quantity from the tissue, and in order to obtain inhibition under the experimental conditions both tissue and yeast cells must be present simultaneously in the medium.

The inhibiting effect of extracts of poppy petals on yeast growth was observed only in media without pantothenic acid (Table 8).

The extract contains a considerable amount of biotin; thus it increases yeast growth on media without biotin. The inhibiting action of the extract does not depend on the content of biotin in the medium. The extracts with antivitamin action are those from materials which were picked alive from the plant. Drying and hot extraction do not deprive the extract of activity. The extract from petals which fell after flowering showed no antivitamin activity.

TABLE 8

Yeast Growth in the Presence of Poppy Petal Extracts

Material	Volume of extract added in ml	Weight of yeast in μg		Substance which determines growth without synthetic pantothenic acid
		Medium with pantothenic acid	Medium without pantothenic acid	
Petals from buds	0.03	407	62	Pantothenic acid
	0.10	646	111	" "
	0.30	526	79	Antagonist
Petals from flowers	0.03	543	30	Pantothenic acid
	0.10	562	102	" "
	0.30	543	18	Antagonist
Fallen petals	0.03	479	51	Pantothenic acid
	0.10	543	153	" "
	0.30	708	195	" "

Pantothenic acid competitively inhibits the action of substances which in their turn are antagonistic to it.

The antagonists of pantothenic acid from the three plants have several general physiological and biochemical properties. The antagonists are found only in living tissues and definite phases in the individual development of the plant. They all block pantothenic acid and it in turn can inhibit the action of all these antagonists. These similarities are not sufficient for the assumption that one and the same substance is active in all these extracts. It is even possible that the antivitamin effect of each extract comes from several substances. In the present work we have not attempted to identify the substances, and so we are limited to a few speculations on this question.

Because of the low stability of the antivitamin, it cannot be one of the fatty acids or hydroxyacids which are toxic for yeasts [13], or an amino acid which competes with a structurally related essential amino acid [14]. The stability of amino acids to acid hydrolysis is higher then that of the antivitamin.

The action of all the amino acids on yeast growth has been frequently tested and none of them inhibits growth [15, 16]. It is known that some amino acids in the presence of small amounts of β-alanine inhibit yeast growth, but only when asparagine and pantothenic acid are excluded from the nutritive medium [17]. A direct test of the action of β-alanine under our experimental conditions (presence of asparagine in the medium) showed that it had no direct or indirect inhibitory action on yeast growth.

In the case of pea extracts pantothenic acid and β-alanine decreased the inhibiting action of the antagonist; asparagine increased it, and so the antagonist could not be β-alanine. For the same reason, the antivitamin in the pea extract could not be identical with phenyl pantothenone one of the analogs and antagonists of pantothenic acid, since this is an antagonist to asparagine [18]. The nature of the physiological action of the antivitamin is very similar to that of derivatives of thiazole [19].

Virtanen and Alfthan [20] found α-ketoadipic acid in sprouting pea seeds; this was not found in any plant. It is interesting that adipic acid is a lower homolog of pimelic acid and the latter is used by some microorganisms for the synthesis of biotin. The probability that the antivitamin of peas is α-ketoadipic acid is small, but it is interesting that different methods show unusual substances in the same type of plant during its growth.

SUMMARY

The sap of pea sprouts, the extract of fresh poppy corolla and live beet tissue inhibit growth of Saccharomyces cerevisiae. The inhibitory effect of the extracts can be reduced by addition to the nutrient medium of increasing doses of pantothenic acid. A preparation has been derived from the sap of pea sprouts exceeding the raw sap in inhibitory action per unit volume by 200-300 times. Small doses of the preparation inhibit, and large doses stimulate yeast growth – a phenomenon only rarely noted in antimetabolites. In the presence of the pea extract asparagine enhances the blockage of pantothenic acid by the antivitamin and retains the capacity to substitute, in part, biotin for yeasts. It is suggested that in all extracts there are present antagonists of pantothenic acid.

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THE USE OF PAPER ELECTROPHORESIS FOR THE STUDY AND SEPARATION OF THE PROTEINS OF MILK FROM HEALTHY COWS AND THOSE WITH BRUCELLOSIS

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Paper electrophoresis has been much used in recent times for the study and separation of proteins of milk and milk whey. A short survey of the literature on this question was given in the recent paper by Sumtsov [1].

The present work is a continuation of our previous study on the separation of casein prepared according to Hammarsten [2]. Our problem is to determine by paper electrophoresis the realtion of the protein content of whey from the milk of cows with brucellosis to that from healthy cows. We have not found any papers on study by electrophoresis of milk proteins from diseased cows in the literature available to us. Aside from study of the relation of the various protein fractions, we have tried to determine the position of the agglutinins in the spectrum of protein fractions from the milk of cows with brucellosis.

METHOD OF STUDY

Choice of Subjects. For experimental animals we used 30 healthy cows and 30 apparently healthy cows which by order of the veterinarian had been isolated because of a positive brucellosis reaction.

Milk samples were collected separately from healthy cows and cows with brucellosis. The cows were in the mid-period of lactation; the average sample was collected from the morning milking.

The milk samples were tested by the method of rapid agglutination of whey and the method of ring precipitation [3]. For the reaction we used a Czechoslovakian antigen ("Bioveta" preparation, Ivanovitse). We took milk samples for electrophoretic study of the proteins from those cows in which both immunological reactions were clearly positive.

Preparation of the Whey. The whey from both healthy cows and those with brucellosis received the same treatment. Before preparation of the whey, the milk fat content was reduced to 0.03% fat. Casein was precipitated from the defatted milk at pH 4.6 with N HCl and then removed by centrifuging; the remaining whey was further filtered. Samples of whey thus prepared were submitted to electrophoretic separation.

Electrophoretic Separation. For the electrophoretic separation of the whey proteins we used a moist chamber, essentially like the chamber of Grassmann and co-workers [4]. By means of a micropipet we deposited 100 μl of uncondensed whey in the form of a wide streak on a dry strip of Whatman No. 1 filter paper 4.5 cm wide. Then the strip of paper was sprayed with a buffer solution, except for the place where the sample had been deposited, dried a little, and pressed against dry filter paper (to remove excess buffer solution). We used the borate buffer (0.005 M $Na_2B_4O_7$ in 0.01 M NaOH), pH 9.5, which we had used successfully in our previous work [2]. The voltage (in v) required in the time (in hours) taken to separate the proteins was 2160 v. The electrophoregram was developed with a 1% solution of bromphenol blue in methanol saturated with mercuric chloride. The dye adsorbed in the paper in places which held no protein was removed by washing with 0.5% aqueous solution of CH_3COOH.

Densitometric Measurement of the Electrophoregram. Some of the electrophoregrams were measured on the densitometer (Fig. 1) described earlier [5]. The colored paper strip was placed in the frame A which moved on a

track over the photoelement B. The movement of the frame was synchronized by a transmitting system with a rotating photographic cylinder C of the Heyrovsky polarograph. The electrical current produced in the photoelement by the action of the light from the electric lamp D which passed through a given portion of the electrophoregram was measured by a mirror galvanometer E whose variations were registered on the photographic paper. The resulting densitometric curve was used to determine the separate protein fractions on the electrophoregram.

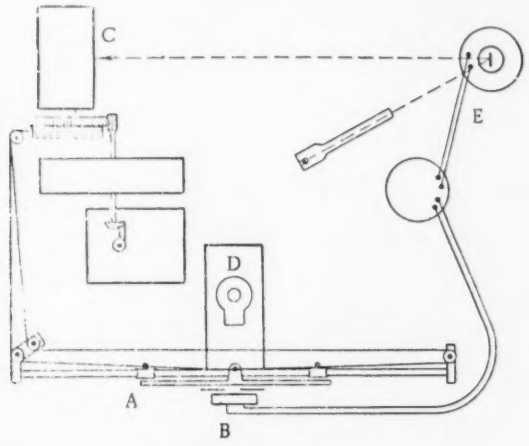

Fig. 1. Plan of the densitometer constructed on the basis of a Heyrovsky polarograph.

Quantitative Estimation of the Electrophoregram. As a rule, determination of the % content of the separate protein fractions was carried out by the photocolorimetric method. The dye was eluted from a paper section of the electrophoregram by half-hour immersion in 5% Na_2CO_3 in 40% methanol. To determine the extinction of the eluate we used a Lang photometric system, Type IV, with an orange filter OD-2.

Study of the Localization of Agglutinins by Paper Electrophoresis. In order to establish the position of the agglutinins on the electrophoregram of whey from cows with brucellosis, 15 ml of whey was dialyzed in a refrigerator against distilled water which was changed three times in 24 hours. After dialysis the whey was concentrated (by passing an air stream over it at room temperature for 2-3 hours) to a volume of about 1.5 ml. Then 150-200 µl of liquid was taken from this concentrated whey and placed on a strip of paper 9 cm wide for electrophoresis. After electrophoretic separation the paper strip was cut in half lengthwise. One half was rapidly colored with bromphenol blue and the other was pressed between two sheets of moist filter paper and placed in the refrigerator for 15 min. The colored strip was then placed next to the uncolored half and the regions of the separate protein fractions were marked on the latter. The parts of the uncolored electrophoregram which contained the separate protein fractions were cut out and cut into small pieces with scissors, and each fraction was eluted with 0.5 ml of phosphate buffer, pH 9.2 [6]. The eluate was used for running the reaction of rapid agglutination as follows: one drop of eluate was placed on a slide and mixed with one drop of brucellosis antigen (length of observation and calculation of the reaction, 3 min).

Besides this method, for comparison of the results we used the method of Berg and Sheiffarth for establishing blood groups [7]. It was carried out as follows: 100 µl of concentrated whey was separated by electrophoresis and then a wide streak of brucellosis antigen colored with fuchsin was added with a brush along the moist strip of paper, placing it either on one edge of the strip, or better, in the middle. The moist strip was placed for 15 min in a thermostat at 40°; then it was removed and the narrow parts noted where diffusion of the antigen away from the original streak did not occur. Then, when we developed the paper strip with bromphenol blue, we determined how the protein fractions agreed with the parts where the antigen was bound.

DISCUSSION

In order to see the effect of brucellosis on the protein fraction of whey, it was first necessary to determine the normal ratios of the protein fractions in whey from healthy cows.

The densitogram and the corresponding electrophoregram (Fig. 2) show that they are basically similar to those described by Wandegaer and Miettinen [8] and by Gal [9]. The fractions which we have designated A and B + C correspond to the fractions usually [8-10] considered to be immunoglobulins, and the fraction E + F corresponds to β-lactoglobulin. Separation of fraction E + F was carried out only in special cases.

Table 1 gives the average values for percent content of the different protein fractions on the protein electophoretogram for 30 healthy cows and 30 with brucellosis; in both cases we used the colorimeter after elution.

We can conclude from these results that no new specific protein fraction is formed in the whey from cows

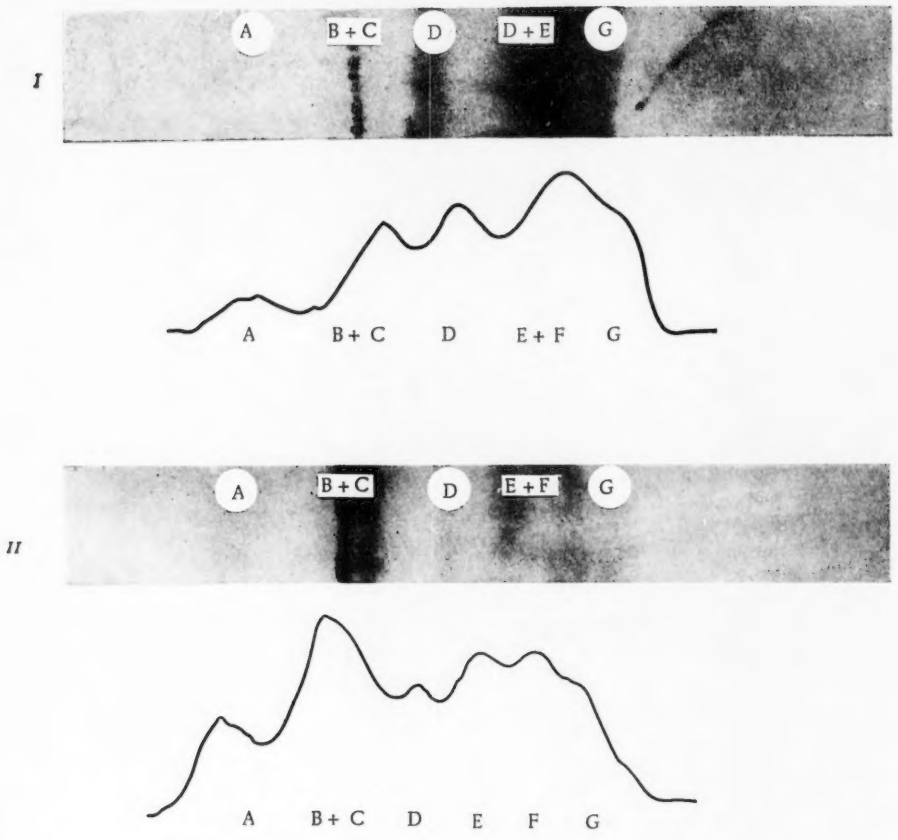

Fig. 2. Electrophoregram of proteins from whey and their densitograms.

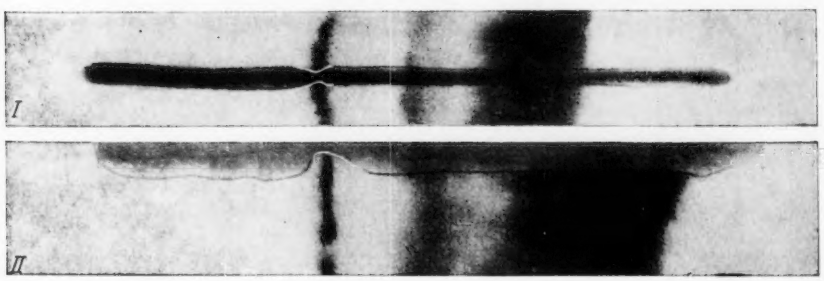

Fig. 3. Localization of agglutinins on the paper electrophoregram.

with brucellosis. However, we show that in the diseased animals there is a small increase in certain fractions (fraction A and fraction B + C) and a simultaneous decrease in fractions D and E + F compared to the quantities of these fractions in whey from healthy cows. The significance of the increase and decrease was also analyzed by statistical methods [11], and by use of the t-test the significance was shown valid to 1% ($p \geq 0.995$). The difference in fraction G in the milk of healthy and diseased cows was slight, while the differences between the other fractions from A to F was significant within the limits of probability given.

TABLE 1

Amount of Separate Protein Fractions in Whey from Healthy Cows and Those with Brucellosis (number of animals in each group: 30; average values for amount given in %)

Animals	Fraction				
	A	B + C	D	E + F	G
Healthy cows	5.7	15.9	22.8	39.2	16.3
Cows with brucellosis	7.6	19.8	20.0	35.3	16.9
t	2.986	3.957	3.612	3.420	0.684
P	≥0.995	≥0.995	≥0.995	≥0.995	Difference uncertain

Further experiments were performed to determine the position of the agglutinins on the electrophoregram prepared from the milk of cows with brucellosis. In the first experiments, when we used unconcentrated whey, the agglutination reaction did not appear clearly enough because of the relatively low concentration of agglutinins; the later samples were concentrated.

TABLE 2

Results of Rapid Agglutination in the Eluate from Different Protein Fractions

Agglutination in fraction		
A	B + C	D – G
–	++++	–
++	++++	–
++	+++	–
++	+++	–
–	++++	–

The results are given in Table 2.

The maximum agglutination reaction with brucellosis antigen is given by fraction B + C. However, fraction A also sometimes shows "residual" agglutination power. We assume that this activity of fraction A is an artifact produced in some cases by a less perfect separation of the large amount of concentrated whey at the site where the sample is added.

In order to show the distribution of the agglutinins on the electrophoregram we later used the application to it of colored brucellosis antigen.

A typical result of these experiments is shown in Fig. 3. Confirmatory tests on 10 samples of concentrated whey carried out by the method which has been described all showed that the whey agglutinins from cows with brucellosis were contained in fraction B + C.

SUMMARY

The method of paper electrophoresis establishes the percent content of protein fractions in the whey from milk of healthy cows and the changes in content of the corresponding fractions in whey from milk of cows with brucellosis. The amount of the immunoglobulin fraction (fraction A and fraction B + C) in cows with brucellosis increases; this was confirmed statistically. This increase is accompanied by a decrease in the relative content of fractions D and E + F, which is also statistically significant.

The position of the agglutinins on the electrophoregram from they whey of cows with brucellosis has been established. Two methods have shown that the agglutinating power occurs chiefly in fraction B + C, that is, in the immunoglobulin.

We express thanks to Candidate in Biological Science B. M. Sumtsov for his comments on the manuscript.

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PRINCIPLES OF STRUCTURE AND ENERGY CORRESPONDENCE IN ENZYMATIC CATALYSIS

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The theory of catalysis has been applied to enzymatic reactions at various stages of their development. Many of these hypotheses have become firmly established in enzymology.

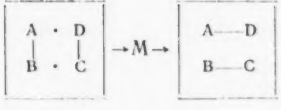
In this connection, the direction initiated by the work of Bredig [1] on colloidal solutions of metals as models of enzymes should be recalled. The theory of intermediate compounds contributed its share thanks to the work of Michaelis [2] and his followers, who proved the existence of intermediate complexes formed between the substrate and enzyme and who, in a number of cases, determined the equilibrium constants for their formation. The adsorption theory of contact catalysis was invoked chiefly to explain the inhibition of enzymes, as, for instance, in the theory of the action of antimetabolites proposed by Woolley [3].

The multiplet theory of catalysis [4] is applied in the present work to reactions taking place under the influence of enzymes and their chemical models. The multiplet theory, the theory of intermediate surface compounds, and the electronic theory of catalysis are mutually supplementary [5], relating to different aspects of the problem. The multiplet theory is concerned mainly with the structural and energy factors in catalysis.

Reaction Indices. Classification of Reactions According to Indices

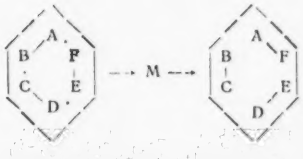
It is now generally accepted that catalysis occurs under the influence of chemical forces. These forces are characterized by chemical bonds which have a definite length (interatomic distance) and energy (dissociation energy). Since the action of chemical forces has a small radius, atoms interact on contact. Therefore it is not whole molecules which must take part in reactions but their individual atoms which, in accordance with the above-mentioned, must come into contact with one another. In a catalytic reaction the reacting atoms must, at a given moment in time, come into contact with the catalyst.

On the basis of these concepts, the multiplet theory [4] postulates the so-called reaction index which includes those atoms which enter into an active intermediate complex M with the catalyst. Enclosed in a frame the doublet index looks as follows:



(1)

and the triplet index:



(2)

The chemical bonds undergo rearrangement as shown above as the result of reaction. Dots indicate active centers which in some reactions are absent.

The principles of structure and energy correspondence deduced by the multiplet theory from the existence of definite bond lengths and bond energies are applicable to the index atoms. This permits indication of a whole series of reaction properties and, in particular, construction of stereochemical models of active complexes [6, 7] and precalculation of the sequence of reactions occurring in different parts of complex organic molecules (for further details on this subject see [8]).

Atoms A, B, C and D may carry substituents which remain unaltered in the course of the reaction but which exert an influence on the length and energy of bonds within the index, and consequently on the rate and the equilibrium constant of the reaction. The theory does not require that all the atoms of the index come in contact with the catalyst at the same time; the existence of stages can be established on the basis of experimental data [9, 10].

The multiplet theory of catalysis has led to the elaboration of a detailed classification based on the chemical criterion – nature of the atoms and bonds in the index. The generalized case of atoms A, B, C, D in the index (1) can be replaced by designations of concrete atoms found in the reactive parts of molecules (H, C, N, O, S, Cl etc.), linked by simple, covalent, double and triple bonds. When special mathematical procedures are applied to this multitude of indices, a complete [4, 11] system of so-called doublet reactions is obtained, and this is a natural classification. The purpose of classification of reaction indices is analogous to the complete system of crystallographic forms as devised by Fedorov. Such a classification of catalytic reactions encompasses all the possible reactions of a given type. On the basis of the elaborated principles, it is possible to extend the system to more complex indices: doublet indices for reactions containing semipolar and ionic bonds, triplet indices for reactions, etc.

The multiplet classification obtained arranges systematically the available experimental material in general agreement with former classifications, establishing finer features of similarity and difference where they were formerly overlooked.

Moreover, the multiplet classification predicted still unknown reactions of which many have subsequently been discovered [4]. Of great importance is the fact that in this classification each type of reaction has its corresponding catalyst.

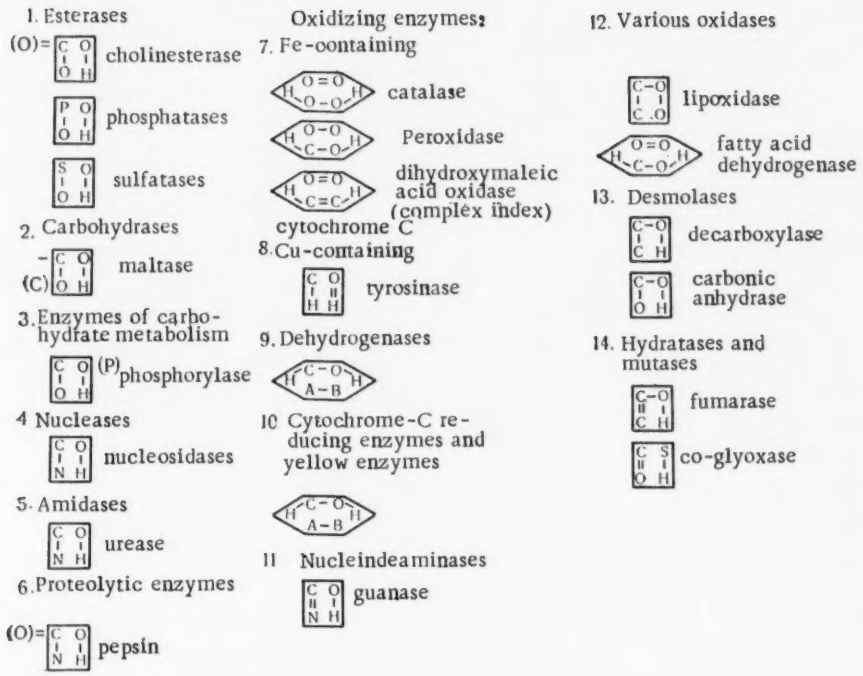
All the catalytic reactions studied by N. D. Zelinskii and his school [12] have been systematized on the basis of this classification. The same principle was applied to the systematization of reactions of catalytic condensation without cleavage as presented in the well-known work of Schwab [13]. All this serves to confirm the great importance of the multiplet classification.

It has been found that the multiplet theory could be extended to the study of enzymatic reactions since enzymes are colloidal, i.e., microheterogeneous catalysts.

Enzymatic reactions have a place of their own in the complete system of doublet and triplet reactions. In accordance with this, all types of enzymatic reactions do in fact have their own catalyst-enzymes whose selectivity is even stronger than that of ordinary catalysis. Since each type of enzymatic reaction is characterized by a definite doublet (or other) index, it occupies a corresponding definite place in the index system. Thus, for example, the cholinesterase index corresponds to the position in the classification [11] designated by the number (index symbol) of II $K^{3}_{43}1$. In mentioning the fact that reactions can be expressed by means of doublet or triplet schemes, it must be pointed out that some reactions do not fit into these schemes. There are many such reactions in organic chemistry and these include, first of all, the majority of molecular rearrangements; the doublet scheme may be applied to some of these only formally. For example, representation of the semidine rearrangement as a doublet index would have entailed bending the benzene ring, which is impossible.

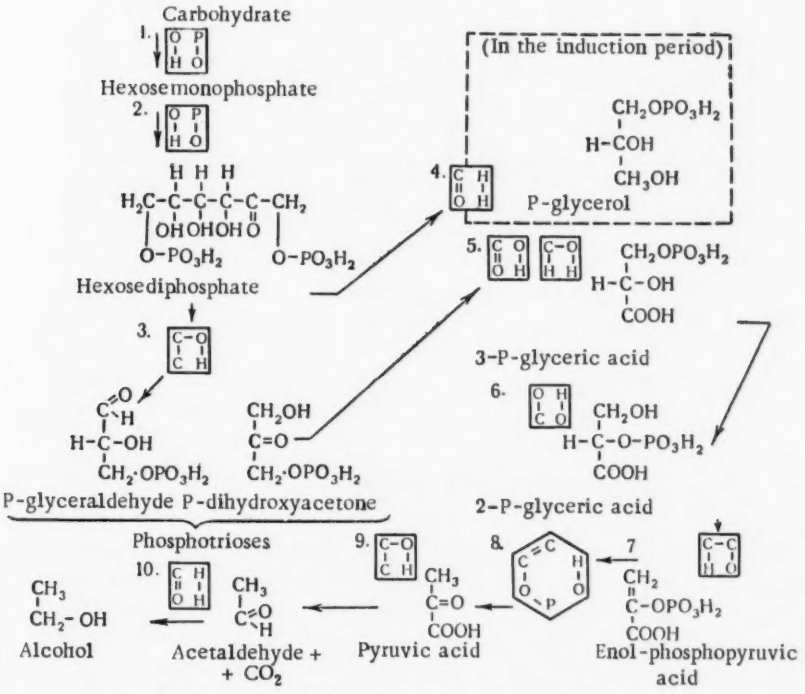
The first step in the study of the action of enzymes from the standpoint of the multiplet theory is the discovery of the position of enzymatic reactions in the classification. This step is extremely important since the chemical affinity of the process is determined by the chemical nature of the reacting atoms included in the reaction index. This should be followed by studies of the structural and energy interrelations of the reacting atoms and the catalyst.

Scheme 1 shows types of enzymes and their indices; it reveals that most of the enzymatic reactions have doublet or triplet indices and that different indices correspond to different types of enzymes. In the indices –C differs from =C, and therefore the indices for esterase and carbohydrase, for example, are different. Indices



Scheme I. Types of enzymes and their indices.

(Some characteristic substituents are shown in brackets)



Scheme II. Sequence of formation of fermentation products.

shown in scheme 1 fall into the multiplet classification naturally; the classification accepted in enzymology [14] thus finds itself in correspondence with the multiplet classification. Classification by indices permits some improvement in the existing classification, e.g., aspartase with index

should be excluded from amidases with their index

In the case of dehydrogenases the hydrogen atoms which are split off reduce another molecule, for example indigo, and the reactions are triplet, as is the decomposition of hydrogen peroxide. Such a complex enzymatic process as fermentation also involves a series of doublet and triplet indices.

Scheme 2 shows the sequence of the formation of fermentation products [15]. Each reaction is accompanied by a representation of its index.

This shows analogies between the multiplet theory and the chemistry of enzymes; indices in scheme 1 correspond to the active groups of substrate in enzyme chemistry or to pharmacodynamic groups; the main valencies participate in them.

The Role of Substituents Outside the Indices

A large number of enzymatic reactions with the same index take place under the influence of various enzymes of more restricted selectivity; this occurs as the result of the influence exerted by "extra-index" substituents.

As in catalysis, substituents exert an influence in enzymatic reactions on the energies of the Q reacting bonds present in the index. According to the multiplet theory, the energy barrier of the reaction (1) equals the energy of formation E' or decomposition E" of the intermediate complex M. Disruption of the bonds A–B and C–D is facilitated by the formation of bonds between the atoms A, B, C, D with catalyst K:

$$E' = -Q_{AB} - Q_{CD} + (Q_{AK} + Q_{BK} + Q_{CK} + Q_{DK}).$$

For decomposition of M the corresponding equation is:

$$E'' = Q_{AD} + Q_{BC} - (Q_{AK} + Q_{BK} + Q_{CK} + Q_{DK}).$$

Introduction of a methyl group next to the group $>C=O$ raises the energy of this bond by 7 kcal. It follows from the equations of the multiplet theory that if the bond $>C=O$ is in the index and instead of a methyl group the substituent is a hydrogen atom and there is no change in the energy of the carbon–catalyst bond, then the energy of formation of the multiplet complex decreases by 7 kcal. and the energy of activation of the reaction by $\frac{3}{4} \times 7 = 5.2$ kcal. According to the Arrhenius equation the rate of reaction constant at 37° then increases 4680-fold [$\log 4680 = 5200/4.57\,(273 + 37)$].

The energy influence of substituents consists of the effect of electron displacement on the energy of bonds within the index.

Structural correspondence of substituents offers a finer adjustment. This is indicated by heterogeneous catalysis of optically active substances. The doublet index group complex is optically inactive in its symmetry, but an optically inactive catalyst deposited on an optically active carrier, e.g., metal on quartz, accelerates selectively the reaction of one optic antipode in a mixture of optical isomers [16]. It may be concluded from this that the asymmetric action of the catalyst or enzyme is concentrated not in the reacting group – index – but in "extra-index" substituents when they are applied to the carrier next to the active center as the result of molecular adsorption. This is in agreement with the conclusion of Kalbunovskii and Patrikeev that the stage of adsorption is asymmetric while the stage of catalysis is symmetric [17].

In order for such position of the substituents, with their large van der Waal's atomic radii, to be possible there must be a sufficient indentation next to the active center where the atoms of the index have smaller valency-chemical radii.

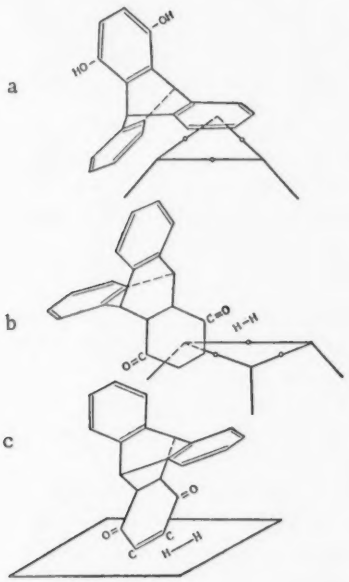


Fig. 1. Hydrogenation of triptycene derivatives on nickel.
a) Hydrogenation of the benzene ring; b) hydrogenation of the carbonyl bond; c) hydrogenation of the olefin bond.

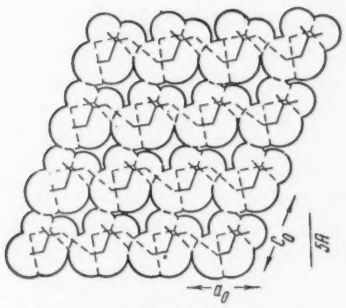


Fig. 2. Surface structure (010) of a crystal of glycine.

Below are some data supporting the above. According to the multiplet theory the atoms of a doublet index lie in one plane. This is confirmed theoretically by calculation on the lines of the theory of absolute rates of reaction: the sum of the exchange energies of s-electrons of four atoms in a chemical reaction will be lowest when the atoms are arranged in one plane [18]. However, a plane index group cannot always be applied to the surface of the catalyst – in complex molecules the "extra-index" substituents may hinder such a juxtaposition sterically. A whole series of such cases has been examined in our laboratory. Especially detailed studies with kinetic measurements were made of the hydrogenation of triptycene derivatives (Balandin, Klabunovskii [6, 7]). It was found that in these cases the reaction took place despite possible steric hindrance. This indicates that the catalytically active centers must be situated not on the plane surface but on projections, in accordance with Taylor's concept of peaks [19], or biographically active centers of Vol'kenshtein [20], and that such disposition of the catalytic centers is explained by the fact that chemical forces are stronger at projections.

The new aspect of our results consists in that such projections must carry small facets at which the index groups dispose themselves, and next to these projections there must be sufficiently deep indentations to accommodate the projecting atoms of the "extra-index" substituents as shown in Fig. 1. Similar results were obtained for dehydrogenation of secondary alcohols and asymmetric diphenylethane [21].

The multiplet theory takes into account that solid catalysts are crystalline in structure and this, too, has to be related to the facets and indentations mentioned above.

Many proteins and enzymes have been isolated in the crystalline form (Northrop et al. [22]). Among the crystalline enzymes are: urease, pepsin, lysozyme, tyrosinase, lecithinase, ribonuclease, ascorbinoxidase, lacticodehydrogenase, peroxidase, catalase from bovine erythrocytes, renin, aldolase, hexokinase etc. In solution the enzymes are present in the colloidal state. This means that their colloidal ions are very small, but, with respect to separate molecules of the substrate, they are still crystals. In this connection we understand by the term crystal a collection of atoms occupying a definite position in space and showing a regular and consistent sequence. In this sense such structures as the fibrous structures of protein put for ward by Pauling should be considered as crystalline.

Recent development in organic crystallography [23, 24] has shown that the molecules in crystals of organic substances are closely packed so that the projections of one molecule are juxtaposed with indentations of another. The configuration of the molecules is found to be such that for two adjacent atoms the distances within one molecule are determined by the sum of atomic radii (i.e., radius of action of valency-chemical forces) and for two touching atoms from two adjacent molecules the distances are determined by the sum of van der Waal's radii (i.e., radii of

the action of molecular forces). Van der Waal's radii or, in other words, intermolecular radii r' are larger than atomic radii r. Both are known (see for example [23] etc.). Thus for C: r' = 1.80, r = 0.77; for H: r' = 1.17, r = = 0.30; for O linked by single bonds: r' = 1.36, r = 0.66; for O ═ : r' = 1.36, r = 0.55; for N: r' = 1.57, r = 0.70; for S: r' = 1.04, r = 0.94 kX.

It is very important that in hydrogen bonds, which strengthen intermolecular attraction markedly, hydrogen does not occupy a separate space in the molecular crystal, the centers of the two atoms linked by hydrogen bonds and present in adjacent molecules being separated by the same distance as in the absence of hydrogen.

The principle of packing of atoms in molecular crystals are illustrated in Fig. 2, which represents the packing of molecules in the crystal of the simplest amino acid, glycine [25]. The structure of apoenzymes, as molecular crystals, must be similar to this except that protein molecules constituting the crystal must be much larger (with regard to protein structure [26]). The considerations expressed above in connection with molecules of organic crystals apply also to the separate part of large protein molecules.

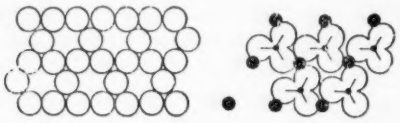


Fig. 3. Scheme of epitaxy of sodium nitrate on the cleavage plane of mica.

The structure of the enzymes considered permits formation of a concept regarding the character of indentations into which the "extra-index" substituents can fit. If a molecule or part of a molecule is extracted from the surface of a molecular crystal an indentation remains which repeats the shape of the removed molecule to the utmost possible degree, viz., at those points at which the removed molecule came in contact with the remaining ones. This indentation can accommodate a similar or structurally very closely related part of a different sort of molecule entering the indentation, part of which is identical with the removed molecule or approaches it in shape. Something in the nature of a surface solid solution is then formed and surface isomorphism must play a special part in this phenomenon, as follows from the considerations mentioned above.

The concept of surface isomorphism must be considered in some more detail. On the one hand, it is related to the concept of space isomorphism in that the substituent atoms must have the configuration and dimensions similar or very closely related to those of the atoms in the molecule of the crystal. On the other hand, surface isomorphism is related to epitaxy (see [27]) or to crystallochemical correspondence (Dankov [28]).

Epitaxy consists of oriented growth of one crystalline solid phase over another. This, as in catalysis, requires structural and energy correspondence. An example of this is the growth of sodium nitrate crystals on the cleavage plane of mica [29] (Fig. 3). Na^+ ions of saltpeter (Fig. 3, right) become arranged over the centers of oxygen cycles (Fig. 3, left) at those points where K^+ ions of mica were found prior to removal by cleavage. Such a phenomenon plays a part in metallurgy; it is a component part in the adsorption–autocatalytic theory of reduction of oxides and ores [30]. Surface isomorphism however, differs from both spatial isomorphism and from epitaxy in that only a part of the molecule, not the whole molecule, need be surface isomorphous. If the other part is directed outward from the surface, then only the remaining part need repeat the shape of the lattice molecules (or their part). The configuration of the projecting part of an isomorphically adsorbed molecule can be most varied, especially owing to free rotation around single bonds. Only in special cases can the projecting part of the molecule have the configuration permitting the formation of a superstructure of crystal lattice made up of the same molecules, when surface adsorption isomorphism becomes epitaxy.

Let us now turn to the experimental data concerning orientation of molecules adsorbed at active centers.

Langmuir's concept regarding the arrangement in adsorption of fatty acids at the surface of water has become familiar; the acids are orientated with their carboxyl groups toward the water.

When the hydrocarbon chain carries a special group, e.g., olefine or amino group, this also comes in contact with the surface and the molecule then lies parallel to the surface of the solution. Studies are made by means of surface tension measurements. Oriented adsorption at liquid surfaces has been investigated in great detail (see for instance [31]). More recent are the interesting investigations by Riedel and his school [32] on chemical reactions in such oriented monomolecular layers at liquid surfaces. These investigations revealed interesting data on orientation. In this case too special groups come in contact with the surface. As an example, lactone formation for γ-hydroxystearic acid [33] can be cited. In the presence of high lateral pressure (high concentration) the molecule

of the latter arranges itself at the surface as shown in Fig. 4, A. Only the carboxyl group is in contact with the acid solution and lactone formation does not take place since the OH group is not in contact with the catalyst (HCl). With small pressures (Fig. 4, B) the hydroxyl group is in contact with the solution and the reaction proceeds.

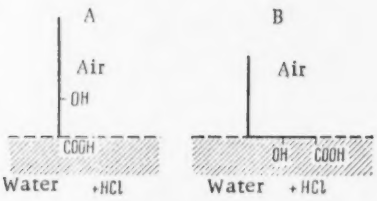


Fig. 4. Lactone formation from γ-hydroxystearic acid at high (A) and low (B) pressures.

Other methods are required for determination of the orientation of molecules during catalysis by solid surfaces.

Recently Schwab and Wandinger [34] attempted to represent the orientation in catalysis of a mixture of D-ethyl alcohols by using statistical treatment of percentage content of HD in the vapor. Their attempt proved unsuccessful since they did not take into account the rapid deuterium exchange of the semihydrogenated form which must be similar to the rapid deuterium exchange of free radicals observed by Voevodskii and Lavrovskaia [35].

More reliable methods for determining the orientation of molecules at active centers of solid (quasihomogeneous) surfaces are: 1) comparison of the chemical structure of the reacting molecules with the energy of activation as well as 2) comparison of the molecular structure with adsorption coefficients found from chemical kinetics.

Results obtained in our laboratory [4] show that during dehydrogenation of many alcohols, amines and hydrocarbons, cyclic and open-chain, the energies of activation are very similar for varous members of a homologous series and that therefore the molecules are oriented with their index group toward the catalyst.

From relative adsorption coefficients determined at various temperatures is found [4] the entropy of displacement of ΔS° and heat of displacement $-\Delta H^\circ$ which for dehydrogenation reactions listed above are found to approach zero in the case of metallic catalysts and are fairly high in the case of oxide catalysts. For metals this confirms the molecular orientation indicated above; for oxide catalysts and alcohols, where hydrogen bond formation is possible, it is found that distribution of "extra-index" substitutents parallelly to the surface of the catalyst is probable. Adsorption of alcohols on aluminosilicates in the precatalytic temperature zone as carried out in Kiselev's laboratory [36] also provides indications that the alcohol molecules are oriented parallel to the surface.

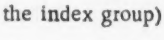
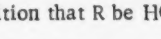
Extensive investigations of Maxted [37, 38] on catalyst poisoning have shown that in adsorption poisoning of platinum catalysts the molecules of mercaptans and thio ethers are oriented with the sulfur atom toward the platinum and the hydrocarbon chains outward. The appearance of a second sulfur atom, as in the case of dithiols, fixes this molecule parallelly to the surface. In this respect the results confirm that if atoms appear in the side chains which are related to the surface and form new linkages (the S-Pt bond in Maxted's work, hydrogen bonds in the case of alcohols with oxide catalysts, hydroxyl-water at liquid surfaces) (see above, experiments of Davies [33]) then the molecule assumes a position parallel to the surface.

Lowering of temperature evidently favors disposition of molecules parallel to the surface because at low temperatures the molecular forces are more pronounced. Raising the temperature leads to increased oscillations of atoms and disruption of the weaker bonds with the surface, with consequent orientation of the side chains perpendicularly to the surface.

The above considerations provide a basis for the fact that at the surface of enzymes active at low temperatures, the side chains of "extra-index" substituents of the substrates are attracted to the surface next to the active centers to which are applied the reacting index atoms.

The scheme of energy levels of molecules in solution (I), molecules at the active center without adsorption of "extra-index" substituents by the carrier (II) and the same with adsorption of the latter by the carrier (III) can be represented as follows (Fig. 5). The energy barrier I-II is greater than I-III, and therefore the reaction is accelerated in I-III. If the adsorbed "extra-index" substituents do not quite fit into their indentations at the surface, the heat of adsorption II-III decreases as the result of increase in the intermolecular distance. The rate of reaction will always be greater when the difference in the levels II-III is maximal when the adsorbed part of the molecule fits the indentation exactly. The given model explains in principle the high selectivity of enzymes illustrated by the ability of spleen cathepsin, acting on amino acids, to hydrolyze peptide chains [39] according to the scheme:

$$\ldots CO-NHCHR \cdot \underline{CO}-\underline{NH} \cdot CHR\ldots + H\underline{OH} = \ldots CO-NH-CHR \cdot CO \cdot OH + H_2N-CHR\ldots$$

(the underscored atoms are in the index group) only on condition that R be HO  $-CH_2=$ or  $-CH_2-$.

Urease hydrolyzes carbamide but not butyl carbamide; the C_4H_9 group cannot fit in where H can.

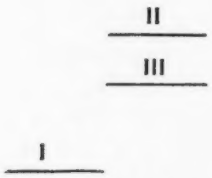


Fig. 5. Energy levels. For designations see text.

In defining the theory more precisely it must be taken into account that there is possibility of the movement of the oriented adsorbed molecules, which together resemble a superficial liquid crystal, along the surface.

In the course of many years of natural selection, enzyme chemistry has established particularly precise structural correspondence between apoenzymes and substituents.

In homogeneous catalysis the molecule of the initial substance can form such a molecular compound with the catalyst that the molecules of both in the complex come in contact not only by means of the reacting atoms but also others. This explains the higher rate of reaction of one of the antipodes with an appropriate optically active catalyst.

Molecular adsorption index substituents – surface isomorphism – also provides an explanation of the particularly high rate of enzymatic reactions. The molecule is maintained at the surface of the enzyme in the position required for the reaction (entropy factor). Adsorption of "extra-index" substitutents diminished the energy barrier of the reaction and the heat of adsorption (energy factor). Taking the energy of the H-bond to be 7kcal. (average) calculation similar to that given above shows the acceleration of the reaction to be 4600-fold. Molecules adsorbed by such groups prove to be more firmly pressed with their index atoms to the active centers of enzyme-catalyst, which is similar to the effect of high pressure.

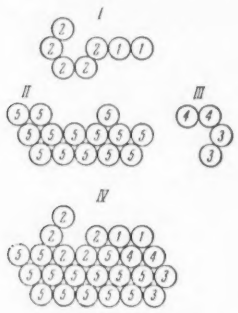


Fig. 6. Scheme of enzyme action. Formation of the intermediate complex in enzyme reaction.
I) Substrate; II) protein part of the enzyme; III) coenzyme; IV) intermediate complex.
1) Atoms in the index group; 2) substituent atoms; 3) coenzyme atoms; 4) atoms of the active part of the coenzyme; 5) atoms of the protein part.

A schematic example of an intermediate complex is given in Fig. 6. In it the substrate (I), the protein part of the enzyme (II) and the coenzyme (III) must be in mutual contact, with good juxtaposition of projections and indentations. For the sake of simplicity, all the atoms in Fig. 6 are similar and no difference is made between the valency-chemical and van-der-Waal's radii. This gives the multiplet theory explanation for Fischer's "lock-and-key" principle.

Enzyme inhibitors act at different stages of the reaction. Strongly adsorbed substances (Hg, HCN, S etc.) block active centers of various enzymes independently of their structure. Conversely, antimetabolites can poison only one out of hundreds of cellular enzymes because their side chains are adsorbed at structurally close indentations of the protein part of the enzymes. Thus the antimetabolite group which is adsorbed at the protein part of the enzyme must be structurally close to the structure of the substrate; the antimetabolite must have stronger tendency to be adsorbed in the index group but can contain various substitutents. Sulfanilamide preparations can serve as an example. The structure of the poisoning group also must not differ greatly from the structure of the index group in the substrate in order to fit the active center of the enzyme. Thus, in p-aminobenzoic acid and sulfanilamide most parts of the molecule are the same ($-NH_2C_6H_5$). The dissimilar groups ($-COOH$ and $-SO_2NH_2$) are structurally similar and their dimensions are close (Fig. 7); the $-SO_2NH_2$ group is more strongly adsorbed.

The new theory of antimetabolism cited above indicates that the theory which regards the antimetabolite as a heterogeneous catalytic poison (Woolley[10]) and the theory pointing out the parallellism between antimetabolism and epitaxy (Erlenmeyer [40]) are not mutually exclusive but supplementary.

Recently the Swiss biochemist Dreiding [41] arrived independently at views concerning structural correspondence in enzymatic catalysis which are close to those developed in the present communication.

SUMMARY

The principles of structural and energy correspondence embodied in the multiplet theory of catalysis can explain the high activity and selectivity of enzyme action. Enzymatic reactions fit into the multiplet theory classification as reactions of the doublet and triplet type.

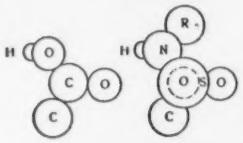


Fig. 7. Configuration of carboxyl groups in aminobenzoic acid and the $-SO_2NH_2$ group in substituted sulfanilamide (the diagram is to scale).

The principle of structural correspondence, as applied to groups of reacting atoms, requires definite relationship between the interatomic distances in the reacting groups and in the parts of enzyme surface with which these groups come in contact during the reaction. The same principle is applicable to substituents at the reacting atoms, leading to the requirement that surface isomorphism be observed between the substituents and those parts of the apoenzyme surface which adsorb them and to which they are applied.

Energy correspondence is manifested in three ways. 1) There must be definite correspondence between the energies of bonds within the reacting group of the substrate and energies of the bonds of the reacting atoms with atoms on the surface of the enzyme. These bond energies depend on the chemical nature of the given atoms. 2) Substituents alter the rate of reaction as a consequence of their influence on the magnitude of bond energies mentioned above, this influence being analogous to the auxochromic effect. 3) The juxtaposition of substituents and atoms of the enzyme surface leads to greater mutual approximation of these and hence to increase of the energy of adsorption of substrate molecules which, in turn, leads to lowering of the energy barrier and considerable acceleration of the rate of enzymatic reaction.

Certain examples are discussed.

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PATHWAYS INVOLVING CARBOHYDRATES IN THE PLASTIC METABOLISM OF CANCER CELLS

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It is now well known that malignant tissues possess greater ability than normal tissues to utilize glucose, the main product of energy transformations, as structural material for the synthesis of the most important high-molecular compounds [1].

The active incorporation of many products formed in the degradation of the hexose molecule into proteins, nucleic acids and lipids of cancer cells occurs only in the presence of two concurrent processes – glycolysis and respiration. Exclusion of glycolysis or respiration lowers sharply the ability of cancer cells to utilize the carbon chain fragments of the carbohydrate for the synthesis or organic compounds [2]. The mechanism explaining this active involvement of carbohydrate in the plastic metabolism of the cancer cell under conditions of aerobic glycolysis can evidently be summed up in successive transformation of the glucose molecule first into lactic acid in the course of the glycolytic cycle and then, in the presence of respiration, its further conversion through dehydrogenation, amination and inclusion in the tricarboxylic acid cycle. The result of these numerous conversions is the formation of a number of amino acids and other precursors from the three-carbon chain derived from glucose; these resultant substances are utilized directly in numerous biological syntheses.

Under conditions of aerobic glycolysis, another possible mechanism leading to the degradation of the glucose molecule cannot be excluded, viz., direct oxidative decomposition of glucose which was first postulated theoretically (Engel'gardt and Barkhash in 1938 [3]). This path, consisting of successive step-wise shortening of the carbon chain, was designated the apotomic decomposition of carbohydrates [4]. The apotomic course creates additional possibilities for the formation of precursors utilized in the synthesis of high-molecular compounds. Lactic acid, the initial product for many further conversions, could be formed not only as the result of dichotomic decomposition of the hexose molecule into two three-carbon fragments, but also in the course of its apotomic breakdown. The existence of direct oxidative decomposition of carbohydrates in tumor tissue has recently been shown in studies using locally labeled 1-C^{14}- and 6-C^{14}-glucose and examination of the radioactivity of expired carbon dioxide [5]. However, studies of the radioactivity of expired carbon dioxide can give only the most general indication of the extent of this mechanism, in no way touching on its significance in the plastic utilization of carbon by one or another tissue.

Isolation and determination of the radioactivity of the products of more profound degradation of the glucose molecule, viz., its three-carbon fragments, can only furnish an impression of the specific importance of both the glycolytic and the apotomic decomposition of the carbohydrate in the formation of precursors for the many biological syntheses in the cell. At the same time, such an investigation permits a more definite answer to be given to the question concerning the mechanism of active utilization of the carbohydrate in the plastic metabolism of the cancer cell in the presence of aerobic glycolysis and respiration.

Results of experiments on the determination of the radioactivity of lactic acid isolated from ascitic cancer cells on incubation of the latter with glucose labeled (C^{14}) in positions one and six are given below. Data are also presented concerning the mechanism of formation of the pentose component of ribonucleic acid by the cancer cells.

Material and Methods

Experiments were carried out on ascitic cells of the Ehrlich carcinoma, suspended in Ringer bicarbonate

solution. Respiration and glycolysis were determined in a Warburg apparatus. The samples were incubated for 80 minutes. 1-C^{14}- and 6-C^{14}-glucose was diluted with unlabeled glucose in such a way that the specific activity of the preparation obtained was equal to $6 \cdot 10^3$ imp/ min per 1 mg.

Isolation of lactic acid. Following precipitation of proteins with concentrated phosphoric acid, lactic acid was extracted from the centrifugate with ether in the course of 12 hours, in a Kutcher* apparatus. Ether was distilled off, the contents dissolved in a small volume of water and applied to chromatographic paper rolled up into a cylinder. The solvent consisted of the following mixture: butyl alcohol – water – formic acid (90 : 45 : 10). Separation was carried out during 40 hours in an ascending flow. The solvent was passed through twice. Bromophenol blue served as developer. After the chromatogram dried an appropriate strip of paper was cut out at the level of the control. Lactic acid was eluted with water. The volume of the eluate was reduced to 1 ml by evaporation. Lactic acid was determined by the Barker and Summerson method [6]. Radioactivity was determined by an end-window counter.

The acid-insoluble precipitate containing proteins, nucleic acids and lipids was washed repeatedly with trichloroacetic acid. After extraction of the lipid fraction, the ribonucleic (RNA) and desoxyribonucleic (DNA) acids were extracted with perchloric acid by the Ogur method [7]. Quantitative estimation of nucleic acids was performed by means of color reactions for the carbohydrate component. Radioactivity counts were made after preliminary neutralization of the samples.

Isolation of pentose from RNA. Samples containing ribonucleic acid were neutralized and subjected to lyophilic drying. The pentose, bound with purines, was freed from ribonucleic acid by hydrolysis with N H_2SO_4 for 1 hour. The hydrolyzate was applied to chromatographic paper with neutralization of the spots with ammonia. A convenient solvent for separation of pentoses from purines and pyrimidine nucleotides was found in 70% ethyl alcohol which had been used earlier for separation of sugars in studies on glycoalkaloids [8]. The R_f value for pentoses is greater than that for purines and pyrimidine nucleotides easily detected by means of the ultrachemiscope. The pentose was developed by an aniline phthalate reagent. The solvent was passed through the paper twice. Seperation was performed in an ascending stream in the course of 40 hours. A strip of paper was cut out at the level of the control after chromatograms were dried. Pentose was eluted with water and the eluate evaporated down to 1 ml. The pentose was estimated quantitatively by the Mejbaum method [9] and its radioactivity was determined.

RESULTS

In the first series of experiments the radioactivity of lactic acid was studied after incubation of ascitic cancer cells with 1-C^{14}-glucose under aerobic and anaerobic conditions (respiration was blocked by KCN). It might have been expected that the values of specific radioactivity of lactic acid isolated from aerobic and anaerobic samples would be close if the dichotomic pathway of glucose conversions under aerobic conditions were the main one for malignant tissues. If the apotomic pathway were predominant the radioactivity of lactic acid in aerobic samples would be markedly lower than that in anaerobic samples as the result of oxidative decarboxylation of the labeled first carbon atom in the glucose molecule.

In our experiments (Table 1, experiments 15, 16, 17) we observed values of specific radioactivity of lactic acid from anaerobic and aerobic samples which were extremely close. In the former case the specific radioactivity was only 5% higher than the corresponding value in the aerobic samples. These experiments show sufficiently clearly that under conditions of aerobiosis the dominant process of glucose catabolism consists of its symmetric division into two 3-carbon compounds.

Comparison of the radioactivity values for lactic acid following incubation of cancer cells aerobically in the presence of 1-C^{14}- and 6-C^{14}-glucose also provides some indication of the relative importance of the dichotomic and apotomic pathways in the formation of three-carbon fragments of glucose. There is no doubt that radioactivity of lactic acid in samples incubated with 1-C^{14}-glucose is characterized by the dichotomic pathway of its transformations, since in apotomic decomposition the radioactive label should not appear in the lactic acid. In samples incubated with 6-C^{14}-glucose the radioactivity of lactic acid is determined by the two pathways of its catabolism. The difference between the values of lactic acid radioactivity in samples incubated with 6-C^{14}- and 1-C^{14}-glucose provides evidence of the relative importance of the direct oxidative pathway in the formation of lactate. The values of specific radioactivity of lactic acid presented indicate clearly that in some experiments these values are almost identical (Table 1, experiments 27, 29), in others this difference does not exceed 10%. It follows from this

* Transliterated from Russian.

that under conditions approaching the physiologic conditions of the cancer cell, i.e., aerobiosis with the presence of glucose in the medium, the conversions undergone by the carbohydrate molecule occur chiefly in accordance with the classic scheme of Embden-Meyerhof. Although the apotomic pathway of glucose decomposition does take place, its part in the formation of the final three-carbon compounds is very insignificant, amounting to 5-10%.

TABLE 1

Radioactivity of Lactic Acid After Incubation of Ascitic Cancer Cells with 1-C^{14}- and 6-C^{14}-Glucose

Specific activity in imp/ minute per 1 μg lactic acid

No. of exp.	1-C^{14}-glucose				No. of exp.	1-C^{14}-glucose		6-C^{14}-glucose	
	Anaerobic		Aerobic			Aerobic			
	Activity in imp/ min	Specific activity	Activity in imp/ min	Specific acticity		Activity in imp/ min	Specific activity	Activity in imp/ min	Specific activity
15	504	5,3	486	5,0	*26*	704	5.4	628	4.8
16	657	5,6	617	5,4	*27*	736	5.6	748	5.7
17	531	5,5	504	5,2	*28*	700	5.4	768	5.9
					29	780	6.0	760	5.8

The three-carbon fragments of glucose are thus formed, in the case of simultaneous coexistence of glycolysis and respiration, almost exclusively through the symmetrical cleavage of the hexose molecule, as is the case in the anaerobic process alone. Nonetheless, intensive utilization of carbohydrate as construction material by cancer cells occurs, as already mentioned, only under conditions of aerobic glycolysis. Our experiments with 1-C^{14}- and 6-C^{14}-glucose also convinced us that incorporation of the label into the cancer cell proteins occurred 10-20 times more intensively under aerobic conditions than under anaerobic conditions (Table 2, experiments 15, 16, 17, 20, 21, 22).

TABLE 2

Incorporation of 1-C^{14}- and 6-C^{14}-Glucose into Proteins of Ascitic Cancer Cells

Radioactivity expressed in imp/ minutes per 10 μg protein

No. exp.	1-C^{14}-glucose		6-C^{14}-glucose		No. exp	1-C^{14}-glucose	6-C^{14}-glucose
	Anaerob.	Aerob	Anaerob.	Aerob.		Aerobic	
15	104	1136			*25*	1124	1104
16	132	1260			*26*	2232	2216
17	120	2408			*27*	2364	2562
20			80	1736	*28*	1688	1828
21			84	1780	*29*	2124	2124
22			160	2000	*30*	1260	1340

The formation of precursors needed for the synthesis of protein, nucleic acids and other compounds evidently occurs in subsequent reactions following dehydrogenation of lactate to pyruvate, with extensive participation of respiratory metabolism. In this connection it could have been expected that incubation of cancer cells under aerobic conditions with 1-C^{14}-and 6-C^{14}-glucose would lead to values of protein radioactivity which would lie close together, since the three-carbon precursors of amino acids formed as the result of symmetric cleavage of hexose are identical not only chemically but also with respect to radioactivity values. Our experiments confirmed this hypothesis. The radioactivity values for proteins in samples incubated with 1-C^{14}- and 6-C^{14}-glucose coincided almost completely in a number of experiments (Table 2, experiments 25, 26, 29) and in others the difference did not exceed 10%.

Similar results were obtained in experiments with DNA. It is known that utilization of carbohydrates in the biosynthesis of DNA involves their participation in the synthesis of purines, pyrimidines and the pentose component.

Compounds participating in the biosynthesis of purines and pyrimidine nucleotides arise from the pyruvate molecule in different ways – on decarboxylation and formation of acetic acid, through inclusion in the tricarboxylic acid cycle and formation of oxaloacetic acid, glutamic acid, glycine etc. The desoxypentose component is formed as the result of condensation of 3- and 2-carbon compounds. In all these various reactions the labeled three-carbon precursor arising on degradation of 1-C^{14}- and 6-C^{14}-glucose is the same. This makes it apparent that incorporation of the label into DNA isolated from cancer cells incubated with 1-C^{14}- and 6-C^{14}-glucose must be approximately the same. Our experiments confirmed this (Table 3).

TABLE 3

Incorporation of 1-C^{14}- and 6-C^{14}-Glucose into DNA and RNA of Ascitic Cancer Cells

Specific activity in imp/ minute per 1 μg DNA and RNA

No. of exp.	DNA				No. of exp.	RNA			
	1-C^{14}-glucose		6-C^{14}-glucose			1-C^{14}-glucose		6-C^{14}-glucose	
	Aerobic					Aerobic			
	Activity in imp/ 10 minutes	Spec. activity	Activity in imp/ 10 minutes	Spec. activity		Activity in imp/ 10 minutes	Spec. activity	Activity in imp/ 10 minutes	Spec. activity
24	920	0.130	816	0.116	*24*	104	0.017	64	0.010
26	944	0.125	948	0.126					
27	704	0.138	640	0.125	*27*	124	0.020	52	0.009
29	768	0.128	776	0.129	*29*	388	0.024	240	0.015
36	868	0.115	720	0.096	*30*	388	0.024	204	0.015

Entirely different results were obtained in experiments in which the utilization of carbohydrate in ribonucleic acid synthesis was studied. Incorporation of the radioactive label into the RNA of samples incubated with 1-C^{14}--glucose proved to be almost twice as intensive as in samples with 6-C^{14}-glucose (Table 3). This result first appeared to be unexpected since tumors show relatively extensive apotomic way of glucose conversion which leads to the formation of pentosephosphate, one of the components of ribonucleic acid. It seemed, in this connection, that incorporation of the label into RNA of samples with 1-C^{14}-glucose would be considerably less than in the case of 6-C^{14}-glucose, owing to incorporation of nonradioactive pentose component. The observed differences in the radioactivity of RNA samples suggested that the synthesis of ribose-5-phosphate in tumors followed some other pathway. Free ribose, as already indicated, was isolated from its other components after hydrolysis of RNA by means of paper chromatography.

TABLE 4

Incorporation of 1-C^{14}- and 6-C^{14}-Glucose into Ribose of Ascitic Cancer Cell Ribonucleic Acid

Specific activity in imp/ minute per 1 μg pentose

No. of experiment	1-C^{14}-glucose		6-C^{44}-glucose	
	Activity in imp/ 40 min	Specific activity	Activity in imp/ 40 min	Specific acitivity
31	756	0.066	376	0.033
35	624	0.069	320	0.036

Two typical experiments are summarized in Table 4. The radioactivity of ribose from samples incubated with 1-C^{14}-glucose is twice that of samples incubated with 6-C^{14}-glucose. The differences obtained can be explained by the existence in tumors of significant transketolase–transaldolase mechanism leading to the formation of ribose-5-phosphate. The triosephosphate formed on symmetric cleavage of glucose condenses with active glycolaldehyde which can arise, as shown by Racker et al. [9], from fructose-6-phosphate. In samples with 1-C^{14}--glucose the ribose formed can thus acquire the label not only from triosephosphate but also from the two-carbon component condensing with it. Hence, although our experiments do not exclude the possibility of ribose formation in tumors by way of the hexosemonophosphate shunt, there is no doubt that the main mechanism responsible for its formation is the transketolase–transaldolase pathway.

DISCUSSION

Analysis of the experimental material suggests that the utilization of carbohydrates in the synthesis of the most important high-molecular compounds occurs in tumor cells with the participation of both anaerobic and aerobic metabolism. The first stages of transformations of the glucose molecule, in the presence of respiration and aerobic glycolysis, occur in accordance with the classic scheme of Embden-Meyerhof i.e., by way of symmetric cleavage of the hexose chain. The share of the apotomic pathway in the formation of lactate constitutes 5, at most 10, %. Our data agree with the results of American investigators who reached the conclusion that 90% and sometimes 98% of the lactate formed in tumors under aerobiotic conditions passes through the dichotomic pathway [11]. At the stage of lactate formation in the cell, i.e., the first stages of carbohydrate catabolism, the formation of precursors for the numerous biological syntheses is very insignificant. When tumor-cell respiration is blocked the utilization of hexose chain fragments for the synthesis of proteins is low, despite the increased formation of lactic acid. The presence of respiratory metabolism exerts a sharply stimulating effect on the utilization of carbohydrates in plastic metabolism. Incorporation of labeled carbon into proteins rises 20-fold compared with anaerobic samples. Evidently, only after dehydrogenation of lactic acid to pyruvate is the broad pathway opened up for further transformations of the three-carbon fragments of hexose in amination reactions and inclusion in the tricarboxylic acid cycle. It is in these reactions, with participation of respiratory metabolism, that the formation of precursors, participating directly in numerous biosyntheses takes place. According to the data reported by a number of workers, the relatively high level of radioactivity seen on incubation of tumor cells with radioactive glucose was found in alanine and glutamic acid [12], as well as in serine and glycine [13]. Our preliminary calculations show that in the course of only 80 minutes' incubation about 0.6% of the tumor-protein carbon is renewed at the expense of the glucose-carbon skeleton. The high level of carbohydrate utilization in the synthesis of nucleic acids and lipids in tumor cells also occurs, as already noted, in the presence of two energy mechanisms [2]. Neither glycolysis nor respiration can ensure separately the formation from carbohydrate of substantial amounts of compounds assimilation by the cell in plastic metabolism. The simultaneous coexistence in the cancer cell of intensive aerobic glycolysis and respiration is apparently of profound biological importance, ensuring maximal utilization in biologic syntheses of intermediate compounds arising in the course of carbohydrate transformation.

The importance of aerobic glycolysis and respiration of tumors is not confined to their participation in the utilization of the carbohydrate carbon skeleton as structural material in the synthetic activity of the cells. There are indications that glycolysis can markedly intensify the synthesis of fatty acids in tumor cells [14]. This is evidently determined not so much by increase of the total energy potential of the cell by inclusion of the glycolytic mechanism as by the importance of intermediate products which appear in the process of carbohydrate catabolism. Thus, not only respiration can increase considerably the intensity of utilization of compounds formed in reactions of the glycolytic cycle in plastic metabolism, but glycolysis, too, can activate markedly the synthesis of fatty acids in the cancer cell.

The extensive literature on tumor metabolism, including the latest work of Warburg [15], deals with the question of aerobic glycolysis and respiration of malignant tissues exclusively in the light of their energy significance in the various biologic syntheses. But these processes participate in anabolic reactions not only as energy donors but also as mechanisms responsible for the formation of enormous quantities of compounds materially included in the structure of the live substance of cancer cells. These additional ways of involving carbohydrate material in the increased synthetic activity of cancer cells may serve as factors accelerating malignant growth.

SUMMARY

The radioactivity of lactic acid following incubation of ascitic cancer cells with 1-C^{14}-glucose under anaerobic conditions exceeds the corresponding value for aerobic samples by 5%. Comparison of specific radioactivity of lactic acid on incubation of ascitic cancer cells with 1-C^{14}- and 6-C^{14}-glucose under aerobic conditions showed close similarity between the values in the two cases. The results obtained suggest that formation of lactic acid by malignant tissues under aerobic conditions occurs along the dichotomic pathway, i.e., symmetric cleavage of the hexose chain, to the extent of 90-95%.

The values of protein radioactivity of ascitic cancer cells after incubation with 1-C^{14}- and 6-C^{14}-glucose under aerobic conditions are similar.

On transition from anaerobic to aerobic conditions, the value of the radioactivity of tumor proteins increases 10–20-fold. It is suggested that the mechanism explaining the active involvement of carbohydrate in plastic meta-

bolism consists of successive conversion of the glucose molecule first in the glycolytic cycle to lactic acid and then, in the presence of respiratory metabolism, of further conversions of the three-carbon product by way of dehydrogenation, amination and inclusion in the tricarboxylic acid cycle.

The radioactivity value for RNA of ascitic cancer cells incubated with 1-C^{14}-glucose under aerobic conditions is twice that for samples with 6-C^{14}-glucose. Similar results were obtained in experiments on isolation of the pentose component of RNA: in samples with 1-C^{14}-glucose the RNA pentose showed twice the specific radioactivity than in samples with 6-C^{14}-glucose. These data indicate that the main way in which the RNA pentose is formed in the tumor cell follows not the apotomic pathway but occurs in reactions of intermolecular migration, most likely, by way of condensation of the two-carbon chain of glycol aldehyde with phosphotriose.

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ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiia	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiia	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Embriologii	Archives of Anatomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiia	Biokhimiia	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiia Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Biull. Eksptl. Biol. i Med.	Biulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Biull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Biulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Eksptl. Khirurg.	Eksperimentalnaia Khirurgiia	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiia i Toksikologiia	Pharmacology and Toxicology
Farmatsiia	Farmatsiia	Pharmacy
Fiziol. Rastenii	Fiziologiia Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenova Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariia	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiia Akademii Nauk SSSR, Seriia Biologicheskaia	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiia Tikhookeanskogo N. I. Instituta Rybnogo Khoziaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiia	Khirurgiia	Surgery
Klin. Med.	Klinicheskaia Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaia Parazitologiia i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaia Radiologiia	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiia	Mikrobiologiia	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhiat. i Psikhogig.	Nevropatologiia, Psikhiatriia i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediia, Travmatologiia i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriia	Pediatriia	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniia Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaia Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journals
Stomatologiia	Stomatologiia	Stomatology
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriia	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics

* LIBRARY OF CONGRESS TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiia	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiia	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiia, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoiuz. Gidrobiol. Obshchestva	Trudy Vsesoiuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoiuz. Inst. Eksptl. Med.	Trudy Vsesoiuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiia	Urologiia	Urology
Uspekhi Biokhimiia	Uspekhi Biokhimiia	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiia	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriia Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriia Biologii i Pochvovedeniia	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiia	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariia	Veterinariia	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniia	Voprosy Pitaniia	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaia Laboratoriia	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiia	Journal of General Biology
Zhur. Vysshei Nerv. Deiatel.	Zhurnal Vysshei Nervnoi Deiatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

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